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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/743162	
INTERNATIONAL APPLICATION NO. PCT/GB99/02158		INTERNATIONAL FILING DATE 6 July 1999		PRIORITY DATE CLAIMED 6 July 1998	
TITLE OF INVENTION POLYKETIDES, THEIR PREPARATION, AND MATERIALS FOR USE THEREIN					
APPLICANT(S) FOR DO/EO/US KELLENBERGER, Johannes et al.					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendemnts has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). Items 11. to 16. below concern document(s) or information included: 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input checked="" type="checkbox"/> Other items or information: Abstract of the Disclosure (1 page) Paper Copy of Sequence Listing (31 pages) Computer-Readable Copy of Sequence Listing The undersigned hereby verifies that the paper copy of the sequence listing and the computer-readable copy of the sequence listing are identical and do not contain any new matter.					

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THE UNITED STATES PATENT AND TRADEMARK OFFICE

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International Filing Date : 6 July 1999
Inventor(s) : Johannes Kellenberger et al.
Title : POLYKETIDES, THEIR
PREPARATION, AND MATERIALS
FOR USE THEREIN

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Assistant Commissioner
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PRELIMINARY AMENDMENT

Dear Sir:

Before calculation of the filing fee, please amend the
above-referenced patent application as follows:

In the Specification:

After the claims, please insert the attached Abstract
of the Disclosure.

In the Claims:

Please amend the claims as follows:

Claim 4, line 1, delete "any preceding claim" and
insert therefor --claim 1--.

Claim 5, line 1, delete "any preceding claim" and

insert therefor --claim 1--.

Claim 6, line 1, delete "any preceding claim" and insert therefor --claim 1--.

Claim 7, line 1, delete "any preceding claim" and insert therefor --claim 1--.

Claim 8, line 1, delete "any preceding claim" and insert therefor --claim 1--.

Claim 9, line 1, delete "any preceding claim" and insert therefor --claim 1--.

Claim 12, line 1, delete "or claim 11".

13. (Amended) A nucleic acid according to [any one of claims 10 to 12] claim 10 wherein at least one of said one or more reductive enzymes is from a different polyketide synthase.

Claim 14, lines 1 and 2, delete "any preceding claim" and insert therefor --claim 1--.

15. (Amended) A host cell transfected, transformed or conjugated with a nucleic acid [or vector] as defined in [any preceding] claim 1.

18. (Amended) A method for producing a nucleic acid encoding a novel polyketide synthase, the method including the steps of:

i. providing a nucleic acid as defined in [any one of claims 1 to 8] claim 1; and

ii. incorporating into said nucleic acid a

nucleic acid sequence which encodes at least one reductive enzyme.

19. (Amended) A method according to claim 18 wherein said nucleic acid sequence encoding at least one reductive enzyme is as defined in [any one of claims 9 to 13] claim 9.

24. (Amended) A method for producing a polyketide, the method including the steps of:

i. providing a fermentation product resulting from the method of claim 20[, or a fermentation product according to any of claims 21-23]; and

ii at least partially purifying a polyketide from said fermentation product.

Please add new claims 28 and 29, as follows:

28. A host cell transfected, transformed or conjugated with a vector as defined in claim 14.

29. A method for producing a polyketide, the method including the steps of:

i. providing a fermentation product which contains a B₁ avermectin substantially free of B₂ avermectins; and

ii. at least partially purifying a B₁ avermectin from said fermentation product.

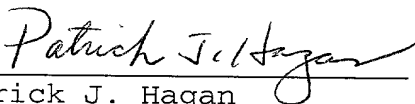
Please cancel claim 27.

REMARKS

The purpose of this Preliminary Amendment is to delete multiple claims dependencies and to add claims directed to preferred embodiments of the invention.

The foregoing amendments do not introduce new matter into the present application, and, therefore should not be deemed objectionable. Entry of the present amendments is respectfully requested.

Respectfully submitted,



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PJH:ksk

09/743162

500 Rec'd PCT/PTO 0 5 JAN 2001

Abstract of the Disclosure

Nucleic acid molecules encoding at least part of a Type I polyketide synthase, and having a polylinker with multiple restriction enzyme sites in place of one or more PKS genes encoding enzymes associated with reduction, optionally further including nucleic acid incorporated into the polylinker, the further nucleic acid encoding one or more reductive enzymes; plasmids incorporating such nucleic acids; host cells transfected with such plasmids; methods relating thereto.

09/743162

POLYKETIDES, THEIR PREPARATION, AND MATERIALS FOR USE
THEREIN

The present invention relates to polyketides, their preparation, and materials for use therein.

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Polyketides are a large and structurally diverse class of natural products that includes many compounds possessing antibiotic or other pharmacological properties, such as erythromycin, tetracyclines, rapamycin, avermectin, polyether ionophores, and FK506.

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In particular, polyketides are abundantly produced by Streptomyces and related actinomycete bacteria. They are synthesised by the repeated stepwise condensation of acylthioesters in a manner analogous to that of fatty acid biosynthesis. The greater structural diversity found among natural polyketides arises from the selection of (usually) acetate or propionate as "starter" or "extender" units; and from the differing degree of processing of the β -keto group observed after each condensation. Examples of processing steps include reduction to β -hydroxyacyl-, reduction followed by dehydration to 2-enoyl-, and complete reduction to the saturated acylthioester. The stereochemical outcome of these processing steps is also specified for each cycle of chain extension.

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The biosynthesis of polyketides is initiated by a group of chain-forming enzymes known as polyketide synthases (PKSs). Two classes of polyketide synthase have been described in actinomycetes. However the novel

- 2 -

polyketides and processes which are the subject of the present invention relate mainly to Type I PKSs, represented by the PKSs for the macrolides erythromycin, rapamycin and avermectin. Type I PKSs contain a different set or "module" of enzymes for each cycle of polyketide chain extension (Cortes, J. et al. Nature (1990) 348:176-178; Donadio, S. et al. Science (1991) 252:675-679; MacNeil, D. J. et al. Gene (1991) 115:119-125; Schwecke, T. et al. Proc.Natl. Acad. Sci. USA (1995) 92:7839-7843 and see e.g. Figure 1 herein, or Figures 2a and 3 of WO98/01546); whereas Type II PKSs are represented by the synthases for aromatic compounds and contain only a single set of enzymatic activities for chain extension. These are re-used as appropriate in successive cycles.

A complete module dictating full reduction contains a ketoacyl-ACP synthase (KS) domain; an acyl carrier protein domain (ACP); an acyl-CoA:ACP acyltransferase (AT) for loading of the extender unit; and a ketoreductase (KR), a dehydratase (DH) and an enoylreductase (ER) domain for accomplishment of the processing of the β -keto group. Since these domains have enzymic activity, they may also be referred to herein as "enzymes", though this is not intended to imply anything about their structural relationship to other PKS domains. Similarly, the nucleic acid sequences encoding such domains may also be referred to as "genes", though this is not intended to imply anything about the presence or otherwise of separate regulatory regions for the different domains of a PKS.

- 3 -

The present invention particularly relates to processes for preparing polyketides by replacing the reductive loop (the segment from the end of the AT to the beginning of the ACP comprising either a KR or a KR and a DH or a KR, a DH and a ER) in a selected module of a Type I polyketide synthase gene cluster by the equivalent segment from the same or from a different PKS gene cluster, or by a mutated or synthetic segment, thereby generating new hybrid polyketide synthases that produce polyketides with different extent of reduction and/or stereochemistry in a predictable way.

For the avoidance of doubt, the term "extension module", as used hereinafter, refers to a set of domains of a Type I PKS, each having enzymic activity, which participate in one cycle of polyketide chain extension. More particularly, an extension module comprises KS, AT, a reductive loop (comprising one or more of KR, DH and ER), and ACP.

Rarely, the reductive loop may include other domains. For example yersiniabacter, which possesses a mixed PKS and polypeptide synthase, possesses a methyl transferase domain.

It has been reported that replacement of the reductive loop of module 2 in DEBS1TE with the equivalent segment of module 3 of the (Type I) erythromycin PKS gene yields a triketide ketolactone when expressed in *S. coelicolor* CH999 (Bedford, D. et al. Chemistry and Biology (1996) 3:827-831).

- 4 -

Similarly, replacement of the reductive loop of module 2 in DEBS1TE with the equivalent segment of module 5 of the erythromycin PKS yields a triketide lactone with the predicted structure and stereochemistry when expressed in *S. coelicolor* CH999 (McDaniel, R. et al. Chemistry and Biology (1997) 4:667-674). On the contrary, when the same experiment was carried out using the reductive loop of module 6 of the erythromycin PKS only a ketolactone could be isolated (McDaniel, R. et al. Chemistry and Biology (1997) 4:667-674).

In a further experiment it has been shown, that the reductive loop of module 2 in a trimodular system comprising the loading domain, the first, second and third extension module and the TE of the ery gene can also be substituted by the equivalent segment of module 4 of the rapamycin PKS comprising a KR and DH domain yielding a tetraketide with the predicted double bond when expressed in *S. coelicolor* CH999 (McDaniel, R. et al. J. Am. Chem. Soc. (1997) 119:4309-4310). In the same system the reductive loop of module 2 has been replaced by the equivalent segment of module 1 of the rapamycin PKS comprising a KR a DH and a ER domain yielding a tetraketide with the predicted oxidation level at C-5 when expressed in *S. coelicolor* CH999 (Kao, C. M. et al. J. Am. Chem. Soc. (1997) 119:11339-11340). On the contrary, when using the corresponding segment of module 4 of the erythromycin PKS gene only a polyketide with a double bond at the relevant position could be observed and not, as one would predict, full reduction (Kao, C. M. et al. J. Am. Chem. Soc. (1997) 119:11339-11340).

- 5 -

In two similar experiments the reductive loop of module 2 in the trimodular system has been substituted by the corresponding segment of module 2 of the rapamycin PKS containing a KR and an inactive DH domain and by the KR domain of module 4 of the rap PKS (the reductive loop of rap module 4 contains a KR and a DH domain). Both
5 constructs are reported to yield a triketide lactone with a different stereochemistry at C-3 (Kao, C. M. et al. J. Am. Chem. Soc. (1998) 120:2478-2479).

10 In all the examples described above the same restriction sites, PstI and XbaI, have been used to join the DNA fragments (the location of the PstI site is identical to the PstI site used in the system described below and the XbaI site is in the same place as the Bsu36I site).

15 A model has been proposed for the structure of the DEB synthase, where the reductive domains form a loop which lies outside the core formed by the KS, AT and the ACP domains (Staunton et al. Nature structural biology (1996)
20 3:188-192). In addition it has been found that DEBS1 is hydrolysed by proteolytic enzymes at specific locations which mark the boundaries of the domains (Aparicio, J. F. et al. J. Biol. Chem. (1994) 269: 8524-8528). These
25 proteolytic sites are found mainly in linker regions and it seems therefore ideal to join the fragments in close neighbourhood to these sites. Examples of this are documented in WO98/01546.

In one aspect the invention provides nucleic acid
30 (particularly DNA) encoding at least part of a Type I polyketide synthase (PKS), said part comprising at least

- 6 -

part of an extension module, wherein the nucleic acid has a polylinker with multiple restriction enzyme sites in place of one or more genes encoding enzymes associated with reduction.

5 In another aspect the invention provides nucleic acid (particularly DNA) encoding at least part of a Type I polyketide synthase, said part comprising at least part of an extension module, wherein the nucleic acid has a polylinker with multiple restriction enzyme sites which
10 connects nucleic acid encoding (at least part of) AT to nucleic acid encoding (at least part of) ACP.

Such nucleic acids may have an additional nucleic acid, which encodes one or more reductive enzymes, inserted
15 into the polylinker as described in more detail below. Such insertion is preferably performed following digestion of the polylinker-containing nucleic acids by two restriction enzymes. In order to provide a choice of insertion sites, the polylinker preferably includes at
20 least three restriction sites, more preferably at least four, and further preferably at least six or eight restriction sites.

The polylinker may be provided by introducing exogenous
25 (usually synthetic) nucleic acid into the Type I PKS-encoding nucleic acid, or may be provided by engineering the existing sequence of the Type I PKS-encoding nucleic acid. For example, to achieve the latter, restriction sites may be engineered (e.g. by site-directed
30 mutagenesis) into sequences up- and/or downstream (preferably both) of where the absent reductive enzyme-

-7-

encoding sequence would normally lie, particularly into sequences which encode polypeptide linkers between the reductive enzyme(s) and adjacent domains.

5 The polylinker desirably includes at least some of the following restriction sites: AvrII, BglII; SnaBI; PstI; SpeI; NsiI; Bsu36I; NheI; and HpaI. More desirably the polylinker includes at least four of these sites.

10 Preferably at least some of the restriction sites included in the polylinker are absent from the remainder of the nucleic acid into which it is incorporated. Desirably at least some of the sites included in the polylinker are uncommon in or absent from naturally occurring nucleic acid sequences which encode reductive enzymes of other (preferably Type I) PKSs. Desirably at 15 least two of the sites are absent from at least about half, more desirably at least about three quarters, of known nucleic acid sequences encoding reductive enzymes of PKSs. Preferably the restriction sites are rich in A and T residues, since PKS genes tend to be rich in G and 20 C residues.

Desirably the nucleic acids of the invention encode a loading module and/or one or more extension modules. 25 More detail concerning varieties of loading modules may be found in our copending international patent application, entitled "Polyketides and their synthesis", filed 29 June 1999.

30 In another aspect the invention provides nucleic acid generally as indicated above but having further nucleic

-8-

acid encoding one or more reductive enzymes (e.g. KR and/or DH and/or ER) inserted into the polylinker. The inserted nucleic acid may encode one or more reductive enzymes of the same polyketide synthase as that of the nucleic acid into which the polylinker is inserted, but from a different extension module. Alternatively the inserted nucleic acid may be exogenous, encoding one or more reductive enzymes from a different natural PKS or fatty acid synthase, or may be synthetic or may be mutated from a naturally occurring nucleic acid which encodes one or more reductive enzymes of a PKS or fatty acid synthase. Preferably, the inserted nucleic acid encodes one or more reductive enzymes from the same or another Type I PKS or fatty acid synthase, but alternatively it may encode one or more reductive enzymes from a Type II PKS or fatty acid synthase.

The genes encoding numerous examples of Type I PKSs have been sequenced and these sequences disclosed in publicly available DNA and protein sequence databases including Genbank, EMBL, and Swissprot. For example the sequences are available for the PKSs governing the synthesis of, respectively, erythromycin (Cortes, J. et al. Nature (1990) 348:176-178; accession number X62569, Donadio, S. et al. Science (1991) 252:675-679; accession number M63677); rapamycin (Schwecke, T. et al. Proc.Natl. Acad. Sci. USA (1995) 92:7839-7843; accession number X86780); rifamycin (August et al. (1998); accession number AF040570); and tylosin (Eli Lilly, accession number U78289), among others. Furthermore, figure 7 herein shows the nucleic acid sequence encoding the first two modules of the avermectin PKS from *S. avermitilis*; this

- 9 -

may be used as an alternative source for the inserts used in certain of the examples.

It is apparent to those skilled in the art that the overall sequence similarity between the nucleic acids encoding comparable domains or modules of different Type I PKSs is sufficiently high, and the domain organisation of different Type I PKSs so consistent between different polyketide-producing microorganisms, that the processes for obtaining novel hybrid polyketides described in the present invention will be generally applicable to all natural modular Type I PKSs or their derivatives.

In further aspects, the present invention provides vectors, such as plasmids or phages (preferably plasmids), including nucleic acids as defined in the above aspects and host cells (particularly of *Streptomyces* species) transfected with such nucleic acids or constructs.

In a still further aspect, the present invention provides polyketide synthases expressible by host cells as defined above. Such polyketide synthases may if desired be isolated from the host cells by routine methods, though it is usually preferable not to do so.

In further aspects the invention provides methods of creating novel functional PKS's and nucleic acids encoding them by means of insertion of nucleic acid encoding reductive enzymes into polylinkers as indicated above; and novel polyketides as produced by such PKS's.

- 10 -

In a still further aspect, the present invention provides novel processes for the specific or preferential production of particular polyketides, using the materials and methods as defined in previous aspects. For example, the present invention provides processes for the generation by direct fermentation of C22-C23 dihydroavermectins, such as ivermectin (see e.g. Examples 25 and 26), and of B1 avermectins substantially free of B2 avermectins (see e.g. Examples 27 and 28).

In another aspect, the present invention provides novel polyketides and novel stereoisomers of polyketides, such as particular polyketides produced in accordance with one or more of the Examples.

In order to enable the exchange of the reductive loop in module 2 of the erythromycin PKS gene in the DEBS1TE system (Cortes J. et al. (1995) 268:1487-1489) a polylinker (multiple cloning site (mcs)) has been inserted in place of the reductive loop of module 2 thereby generating a minimal module comprising a KS, an AT and an ACP. (This system is still functional and produces a ketolactone (see examples 2 and 4).) The mcs contains unique recognition sites for 9 restriction enzymes.

These new restriction sites are situated partly in DNA encoding a linker region near positions where the polyketide synthase is hydrolysed by proteolytic enzymes (vide supra). While some of the restriction sites lie in DNA encoding regions of low homology, others are situated in DNA encoding highly conserved regions (Figure 1). The

- 11 -

introduction of recognition sites for the enzymes AvrII, BglII, Bsu36I and NheI does not change the amino acid sequence in DEBS module 2. In the other five cases (SnaBI, PstI, SpeI, Nsi, HpaI) the amino acid sequence is changed (Figure 2). These changes do not affect the activity of the protein (see example 6).

Because two of the restriction sites cover the same bases it was decided to construct two plasmids containing different mcs (pJLK114 and pJLK117).

The use of an mcs offers the following advantages over a single restriction site on each side of the reductive loop:

- 1) suitable positions to join the DNA fragments (20 different combinations) can be chosen for every different reductive loop thereby avoiding unfavourable changes in the amino acid sequence
- 2) enzymes that cut within the loop can be avoided; and
- 3) loop insertion may be performed in a combinatorial way.

The present inventors have made the further surprising discovery that different results may be obtained using the same polylinker-containing nucleic acid and the same nucleic acid encoding one or more reductive enzymes, when the nucleic acid encoding one or more reductive enzymes is incorporated at different sites in the polylinker.

- 12 -

For example, in Examples 7 and 8, the reductive loop of the rapamycin module 13 was inserted into ery module 2 to bring about complete reduction of the polyketide chain as the outcome of the second extension module. The desired triketide lactone products were obtained in good yield.

5 However, in Examples 37 and 38, the same reductive loop, or set of domains, from rap module 13 was inserted into essentially the same position in ery module 2 as in examples 7 and 8, save that different restriction sites of the polylinker were used (AvrII and HpaI instead of
10 BglII and NsiI) and significant amounts of by-products were obtained. Such by-products included triketide lactones in which C-3 was either keto or hydroxy, showing that simply altering the sites used for swapping the reductive loop made the difference between obtaining the
15 desired product and obtaining an undesirable mixture of the desired product with the products of incomplete reduction.

Similarly, in Examples 31 and 32, when the sites PstI and
20 Bsu36I were used to insert the reductive domains of avermectin module 1 (plasmid pGMS2) in place of the reductive loop of ery module 2, the expected product was produced, but also a substantial amount of ketolactone. In the experiment of Examples 29 and 30, when the sites
25 BglII and NheI were used (plasmid pJLK30) hardly any ketolactone byproduct was produced, although the amounts of lactone were in a similar range in each case.

When, entirely analogously to the Examples 29 and 30, in
30 Example 14 the same sites BglII and NheI were used to replace the reductive loop of ery module 2 with the reductive loop of tylosin module 1 (plasmid pJLK35), the

- 13 -

same target triketide lactones were produced as in Examples 30 and 32 but with much higher yield, albeit accompanied by some ketolactone, demonstrating that different reductive loops may be most advantageously inserted into different restriction sites.

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In Examples 33 and 34, when the sites BglII and NheI were used to insert the reductive domains of avermectin module 2 (plasmid pJLK31) the expected products were produced as the major products. In the experiment of Examples 35 and 10 36, when the sites SnaBI and Bsu36I were used (plasmid pGMS4) only trace amounts of a triketide lactone mixture could be obtained.

Thus, the present invention provides the opportunity, 15 should the desired and predicted products not be obtained when a particular reductive loop is inserted into a particular PKS, of simple adjustment of the insertion site by use of different restriction enzymes having sites in the polylinker. As demonstrated by the above 20 comparative examples, such readjustment can dramatically affect the outcome and yield of polyketide synthesis.

Example 1

25 Construction of plasmid pJLK114

Plasmid pJLK114 is a pCJR24 based plasmid containing a PKS gene comprising the ery loading module, the first and the second extension modules of the ery PKS and the ery 30 chain-terminating thioesterase except that the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module has been substituted by a synthetic oligonucleotide

- 14 -

linker containing the recognition sites of the following restriction enzymes: AvrII, BglII, SnaBI, PstI, SpeI, NsiI, Bsu36I and HpaI. It was constructed via several intermediate plasmids as follows (Figure 3).

5 Construction of plasmid pJLK02

The approximately 1.47 kbp DNA fragment of the eryAI gene of *S. erythraea* was amplified by PCR using as primers the synthetic oligonucleotides:

10 5'-TACCTAGGCCGGGCCGGACTGGTCGACCTGCCGGGTT-3' and
5'-ATGTTAACCGGTTCGCGCAGGCTCTCCGTCT-3' and plasmid pNTEP2
(Oliynyk, M. *et al.*, Chemistry and Biology (1996) 3:833-
839; WO98/01546) as template. The PCR product was treated
with T4 polynucleotide kinase and then ligated with
15 plasmid pUC18, which had been linearised by digestion
with SmaI and then treated with alkaline phosphatase. The
ligation mixture was used to transform electrocompetent
E. coli DH10B cells and individual colonies were checked
for their plasmid content. The desired plasmid pJLK02 was
20 identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK03

25 The approximately 1.12 kbp DNA fragment of the eryAI gene
of *S. erythraea* was amplified by PCR using as primers the
synthetic oligonucleotides:

5'-ATGTTAACGGGTCTGCCGCTGCCGAGCGGAC-3' and
5'-CTTCTAGACTATGAATTCCTCCGCCAGC-3' and plasmid pNTEPH
as template. The PCR product was treated with T4
30 polynucleotide kinase and then ligated with plasmid
pUC18, which had been linearised by digestion with SmaI
and then treated with alkaline phosphatase. The ligation
mixture was used to transform electrocompetent *E. coli*

- 15 -

DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK03 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK04

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Plasmid pJLK02 was digested with PstI and HpaI and the 1.47 kbp insert was ligated with plasmid pJLK03 which had been digested with PstI and HpaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK04 was identified by its restriction pattern.

Construction of plasmid pJLK05

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Plasmid pJLK01 (PCT/GB97/01819) was digested with PstI and AvrII and the 460 bp insert was ligated with plasmid pJLK04 which had been digested with PstI and AvrII. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK05 was identified by its restriction pattern.

Construction of plasmid pJLK07

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Plasmid pJLK05 was digested with ScaI and XbaI and plasmid pNTEP2 was digested with NdeI and ScaI and these two fragments were ligated with plasmid pCJR24 which had been digested with NdeI and XbaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK07 was identified by its restriction pattern.

- 16 -

Construction of plasmid pJLK114

The two synthetic oligonucleotides Plf and Plb (Figure 4) were each dissolved in TE-buffer. 10 μ l of each solution (0.5nmol/ μ l) were mixed and heated for 2 minutes to 65C and then slowly cooled down to room temperature. Plasmid pJLK07 was digested with AvrII and HpaI and ligated with the annealed oligonucleotides. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK114 was identified by its restriction pattern.

Example 2

Use of plasmid pJLK114 for construction of *S. erythraea* JC2/pJLK114 and the production of TKL derivatives

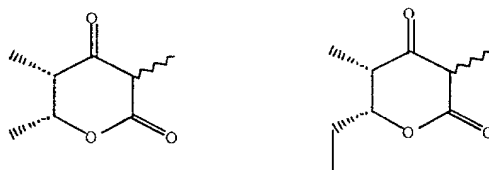
Approximately 5 μ g plasmid pJLK114 were used to transform protoplasts of *S. erythraea* JC2 (strain deposited as No. NCIMB 40802. WO98/01546.) and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE gene. JC2/pJLK114 was plated onto SM3 agar (5.0 g glucose, 50.0 g MD30E maltodextrin, 25.0 g Arkasoy soya flour, 3.0 g molasses (beet), 0.25 g K_2HPO_4 , 2.5 g $CaCO_3$, 22.0 g agar distilled water to 1 litre pH=7.0) containing 50 μ g/ml thiostrepton and allowed to grow for twelve days at 30°C. 1 cm² (500 μ l) of the plate was homogenised and extracted with a mixture of 1.2 ml ethyl acetate and 20 μ l formic acid. The solvent was decanted and removed by evaporation and the residue dissolved in

- 17 -

methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified as (4S, 5R)-5-hydroxy-2,4-dimethyl-3-oxo-n-hexanoic acid- δ -lactone and as (4S, 5R)-5-hydroxy-2,4-dimethyl-3-oxo-n-heptanoic acid- δ -lactone.

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Example 3

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Construction of plasmid pJLK117

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Plasmid pJLK117 is a pCJR24 based plasmid containing a PKS gene comprising the ery loading module, the first and the second extension modules of the ery PKS and the ery chain-terminating thioesterase except that the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module has been substituted by a synthetic oligonucleotide linker containing the recognition sites of the following restriction enzymes. AvrII, BglII, SnaBI, PstI, SpeI, NsiI, Bsu36I and NheI.

30

It was constructed via several intermediate plasmids as follows (Figure 3).

Construction of plasmid pJLK115

Plasmid pJLK114 was digested with NdeI and XbaI and the

- 18 -

approximately 9.9 kbp insert was ligated with plasmid pUC18 which had been digested with NdeI and XbaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK115 was identified by its restriction pattern.

Construction of plasmid pJLK116

Plasmid pJLK13 (PCT/GB97/01819) was digested with Bsu36I and XbaI and the 1.1 kbp fragment was ligated with plasmid pJLK115 which had been digested with Bsu36I and XbaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK116 was identified by its restriction pattern.

Construction of plasmid pJLK117

Plasmid pJLK116 was digested with NdeI and XbaI and the 9.9 kbp fragment was ligated with plasmid pCJR24 which had been digested with NdeI and XbaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK117 was identified by its restriction pattern.

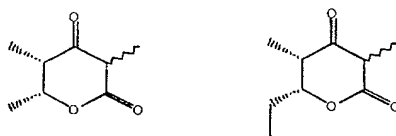
Example 4

Use of plasmid pJLK117 for construction of *S. erythraea* JC2/pJLK117 and the production of TKL derivatives

Approximately 5 μ g plasmid pJLK117 were used to transform

- 19 -

protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE. JC2/pJLK117 was plated onto SM3 agar
5 containing 50 μ g/ml thiostrepton and allowed to grow for twelve days at 30°C. 1 cm² (0.5 ml) of the plate was homogenised and extracted with a mixture of 1.2 ml ethyl acetate and 20 μ l formic acid. The solvent was decanted and removed by evaporation and the residue dissolved in
10 methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified as (4S, 5R)-5-hydroxy-2,4-dimethyl-3-oxo-n-hexanoic acid- δ -lactone and as (4S, 5R)-5-hydroxy-2,4-dimethyl-3-oxo-n-heptanoic acid- δ -lactone.



Example 5

Construction of plasmid pJLK25

Plasmid pJLK25 is a pJLK114 based plasmid except that the DNA fragment encoding the reductive loop of the second module of the erythromycin PKS gene has been inserted into the mcs.

It was constructed via several intermediate plasmids as follows.

- 20 -

Construction of plasmid pJLK118

The approximately 1.4 kbp DNA fragment of the eryAI gene of *S. erythraea* encoding the reductive loop of module 2₁ was amplified by PCR using as primers the synthetic

5'-ATACTAGTCCTCGTGACGAGCTCGACGG-3' and

5'-TAATGCATCCGTTCTCCGGCCCGCTCGCT-3' and pNTEP2 as

template. The PCR product was treated with T4

polynucleotide kinase and then ligated with plasmid

pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli*

DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK118 was

identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK23

Plasmid pJLK118 was digested with SpeI and NsiI and the 1.4 kbp fragment was ligated with plasmid pJLK115 which had been digested with SpeI and NsiI. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK23 was identified by its restriction pattern.

Construction of plasmid pJLK25

Plasmid pJLK23 was digested with NdeI and XbaI and the approximately 11.2 kbp fragment was ligated with plasmid pCJR24 which had been digested with NdeI and XbaI. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked

- 21 -

for their plasmid content. The desired plasmid pJLK25 was identified by its restriction pattern.

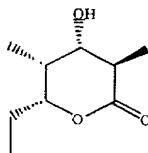
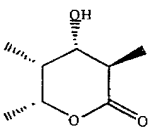
Example 6

- 5 Use of plasmid pJLK25 for construction of *S. erythraea* JC2/pJLK25 and the production of triketides

Approximately 5 μ g plasmid pJLK25 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE. JC2/pJLK25 was plated onto SM3 agar containing 50 μ g/ml thiostrepton and allowed to grow for twelve days at 30°C. 1 cm² (0.5 ml) of the plate was homogenised and extracted with a mixture of 1.2 ml ethyl acetate and 20 μ l formic acid. The solvent was decanted and removed by evaporation and the residue dissolved in methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified (by comparison with authentic material) as (2R, 3S, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-hexanoic acid δ -lactone and as (2R, 3S, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-heptanoic acid δ -lactone.

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- 22 -

Example 7**Construction of plasmid pJLK28**

5 Plasmid pJLK28 is a pJLK117 based plasmid except that the DNA fragment encoding the reductive loop of module 13 of the rap PKS has been inserted into the mcs. It was constructed via several intermediate plasmids as follows. (Figure 5)

10

Construction of plasmid pJLK120

The approximately 3.2 kbp DNA segment of the rapC gene of *S. hygrosopicus* encoding the reductive loop of module 13 was amplified by PCR using as primers the synthetic oligonucleotides:
5'-TAAGATCTTCCGACCTACGCCTTCCAAC-3' and
5'-TAATGCATCGACCTCGTTGCGTGCCGCGGT-3' and cosmid cos 31 (Schwecke, T. et al. (1995) Proc. Natl. Acad. Sci. USA 92:7839-7843) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK120 was identified by its restriction pattern and DNA sequencing.

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Construction of plasmid pJLK28

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Plasmid pJLK120 was digested with BglII and NsiI and the 3.2 kbp fragment was ligated with plasmid pJLK117 which had been digested with BglII and NsiI. The ligation

- 23 -

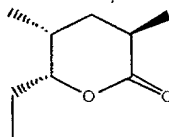
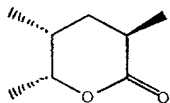
mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK28 was identified by its restriction pattern.

5 Example 8

Use of plasmid pJLK28 for construction of JC2/pJLK28 and the production of triketides

10 Approximately 5 μ g plasmid pJLK28 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE. JC2/pJLK28 was plated onto SM3 agar
15 containing 50 μ g/ml thiostrepton and allowed to grow for twelve days at 30°C. 1 cm² (0.5 ml) of the plate was homogenised and extracted with a mixture of 1.2 ml ethyl acetate and 20 μ l formic acid. The solvent was decanted and removed by evaporation and the residue dissolved in
20 methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified (by comparison with authentic material) as
25 (2R, 4S, 5R)-2,4-dimethyl-5-hydroxy-n-hexanoic acid δ -lactone and as (2R, 4S, 5R)-2,4-dimethyl-5-hydroxy-n-heptanoic acid δ -lactone.

30.



Example 9

Construction of plasmid pJLK41

Plasmid pJLK41 is a pJLK117 based plasmid except that the DNA fragment encoding the reductive loop of module 4 of the ery PKS has been inserted into the mcs. It was constructed via several intermediate plasmids as follows. (Figure 5)

Construction of plasmid pJLK32.3

The approximately 3.2 kbp DNA segment of the eryAII gene of *S. erythraea* encoding the reductive loop of module 4 was amplified by PCR using as primers the synthetic oligonucleotides:

5'-ATAGATCTGCCTACGTACCCGTTCTGAACACCAGCGCTTC-3' and

5'-ATCCTCAGGTTCTGGCCCTGCCGCTCGGCCTGCCCCGGCGGCGCAGCTT-3'

and cosmid cos4B (cosmid containing the erythromycin PKS) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK32.3 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK38

Plasmid pJLK32.3 was digested with BglII and Bsu36I and the 3.2 kbp fragment was ligated with plasmid pJLK116

- 25 -

which had been digested with BglII and Bsu36I. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK38 was identified by its restriction pattern.

5

Construction of plasmid pJLK41

Plasmid pJLK38 was digested with NdeI and XbaI and the approximately 13 kbp fragment was ligated with plasmid pCJR24 which had been digested with NdeI and XbaI. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK41 was identified by its restriction pattern.

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Example 10

Use of plasmid pJLK41 for construction of JC2/pJLK41 and the production of triketides

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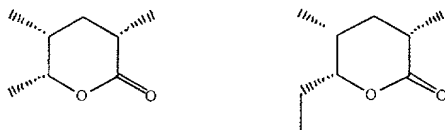
Approximately 5 μ g plasmid pJLK41 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE. JC2/pJLK41 was plated onto SM3 agar containing 50 μ g/ml thiostrepton and allowed to grow for twelve days at 30°C. 1 cm² (0.5 ml) of the plate was homogenised and extracted with a mixture of 1.2 ml ethyl acetate and 20 μ l formic acid. The solvent was decanted and removed by evaporation and the residue dissolved in methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified (by

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- 26 -

comparison with authentic material) as
 (2S, 4S, 5R)-2,4-dimethyl-5-hydroxy-n-hexanoic acid δ -
 lactone and as (2S, 4S, 5R)-2,4-dimethyl-5-hydroxy-n-
 heptanoic acid δ -lactone.

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Example 11

Construction of plasmid pJLK29

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Plasmid pJLK29 is a pJLK117 based plasmid except that the
 DNA fragment encoding the reductive loop of module 10 of
 the rap PKS has been inserted into the mcs. It was
 constructed via several intermediate plasmids as follows.
 (Figure 5)

20

Construction of plasmid pJLK121.1

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The approximately 2.2 kbp DNA segment of the rapB gene of
S. hygroscopicus encoding the reductive loop of module 10
 was amplified by PCR using as primers the synthetic
 oligonucleotides:

5'-TAAGATCTTCCGACGTACGCGTTCCAGC-3' and

5'-ATGCTAGCCACTGCGCCGACGAATCACCGGTGG-3' and as template an

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approximately 7 kbp fragment, which has been obtained by
 digestion of cosmid cos 26 (Schwecke, T. et al. (1995)
 Proc. Natl. Acad. Sci. USA 92:7839-7843) with ScaI and
 SphI. The PCR product was treated with T4 polynucleotide

- 27 -

kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK121.1 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK29

Plasmid pJLK121.1 was digested with BglII and NheI and the 2.2 kbp fragment was ligated with plasmid pJLK117 which had been digested with BglII and NheI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK29 was identified by its restriction pattern.

Example 12

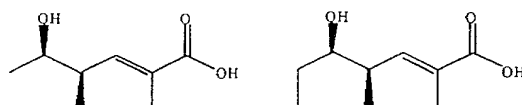
Use of plasmid pJLK29 for construction of *S. erythraea* JC2/pJLK29 and the production of triketides

Approximately 5 μ g plasmid pJLK29 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE. JC2/pJLK29 was used to inoculate 30 ml of SM3 medium containing 5 μ g/ml thiostrepton in a 250 ml flask with a single spring to reduce clumping, shaken at 300 rpm and at 30°C. After 8 days the broth was centrifuged, the supernatant adjusted to pH 3 and extracted three times with an equal volume of ethyl acetate. The solvent was

- 28 -

removed by evaporation and the residue dissolved in methanol and analysed by HPLC and electrospray mass spectroscopy and, after conversion to the methyl ester with trimethylsilyl-diazomethane by GC/MS. The major products were identified (by comparison with authentic material) as

(4S, 5R)- 5-hydroxy-2,4-dimethyl-n-hex-2-enoic acid and as (4S, 5R)-5-hydroxy-2,4-dimethyl0-n-hept-2-enoic acid.



Example 13

Construction of plasmid pJLK35

Plasmid pJLK35 is a pJLK117 based plasmid except that the DNA fragment encoding the reductive loop of module 1 of the tylosin PKS has been inserted into the mcs. It was constructed via several intermediate plasmids as follows. (Figure 5)

Construction of plasmid pJLK33.1

The approximately 1.6 kbp DNA segment of the tylosin PKS gene of *S. fradiae* encoding the reductive loop of module 1 was amplified by PCR using as primers the synthetic

- 29 -

oligonucleotides:

5'-TAAGATCTCCCTACGTACCCCTTCAACCAC-3' and

5'-GCTAGCCGCCGCGCCAGCTCGGGC-3' and cosmid 6T (cosmid containing the tylosin-producing PKS genes) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK33.1 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK35

Plasmid pJLK33.1 was digested with BglII and NheI and the 1.6 kbp fragment was ligated with plasmid pJLK117 which had been digested with BglII and NheI. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK35 was identified by its restriction pattern.

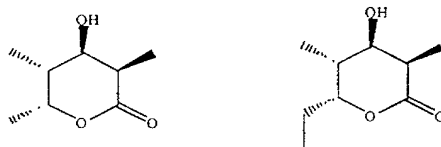
Example 14

Use of plasmid pJLK35 for construction of *S. erythraea* JC2/pJLK35 and the production of triketides

Approximately 5 μ g plasmid pJLK35 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE. JC2/pJLK35 was plated onto SM3 agar

- 30 -

containing 50 $\mu\text{g/ml}$ thiostrepton and allowed to grow for twelve days at 30°C. 1 cm² (0.5 ml) of the plate was homogenised and extracted with a mixture of 1.2 ml ethyl acetate and 20 μl formic acid. The solvent was decanted and removed by evaporation and the residue dissolved in methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified (by comparison with authentic material) as (2R, 3R, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-hexanoic acid δ -lactone and as (2R, 3R, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-heptanoic acid δ -lactone.



Example 15

Construction of plasmid pRIF7

Plasmid pRIF7 is a pJLK117 based plasmid except that the DNA fragment encoding the reductive loop of module 7 of the rifamycin PKS has been inserted into the mcs. It was constructed via several intermediate plasmids as follows. (Figure 5)

Construction of plasmid pUCRIF7

The approximately 2.1 kbp DNA segment of the rifamycin PKS gene of *Amycolatopsis mediterranei* encoding the reductive loop of module 7 was amplified by PCR using as primers the

- 31 -

synthetic oligonucleotides:

5'-CCTACGTACGCCTTCGACCACCAGCACTT-3' and

5'-CGGCTAGCGGGCGTTCCAGGCCGCGTCCT and cosmid 6 (cosmid
starting at 35727 and going beyond 76199, numbers
according to accession number AF040570) as template. The
5 PCR product was treated with T4 polynucleotide kinase and
then ligated with plasmid pUC18, which had been linearised
by digestion with SmaI and then treated with alkaline
phosphatase. The ligation mixture was used to transform
electrocompetent E. coli DH10B cells and individual
10 colonies were checked for their plasmid content. The
desired plasmid pUCRIF7 was identified by its restriction
pattern and DNA sequencing.

Construction of plasmid pRIF7

15 Plasmid pUCRIF7 was digested with SnaBI and NheI and the
2.1 kbp fragment was ligated with plasmid pJLK117 which
had been digested with SnaBI and NheI. The ligation
mixture was used to transform electrocompetent E. coli
20 DH10B cells and individual colonies were checked for their
plasmid content. The desired plasmid pRIF7 was identified
by its restriction pattern.

Example 16

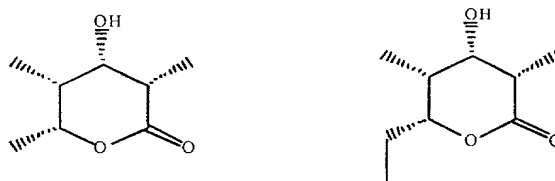
25 Use of plasmid pRIF7 for construction of S. erythraea
JC2/pRIF7 and the production of triketides

30 Approximately 5 μ g plasmid pRIF7 were used to transform
protoplasts of S. erythraea JC2 and stable thiostrepton
resistant colonies were isolated. From several colonies
total DNA was obtained and analysed by Southern blot
hybridisation, to confirm that the plasmid has integrated

- 32 -

into the TE. JC2/pRIF7 was plated onto SM3 agar containing 50 $\mu\text{g/ml}$ thiostrepton and allowed to grow for twelve days at 30°C. 1 cm^2 of the plate was homogenised and extracted with a mixture of 1.2 ml ethyl acetate and 20 μl formic acid. The solvent was decanted and removed by evaporation and the residue dissolved in methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified (by comparison with authentic material) as

(2S, 3S, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-hexanoic acid δ -lactone and as (2R, 3R, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-heptanoic acid δ -lactone.



Example 17

Construction of plasmid pJLK52

Plasmid pJLK52 is a pJLK35 based plasmid containing a PKS gene comprising the ery loading module, the first, the second and the third extension modules of the ery cluster and the ery chain-terminating thioesterase except that the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module

- 33 -

has been substituted by the equivalent segment of module 1 of the tylosin PKS.

It was constructed via several intermediate plasmids as follows.

5 Construction of plasmid pJLK50

The approximately 6.1 kbp DNA segment of the erythromycin PKS gene cluster of *S. erythraea* encoding the DNA fragment from the beginning of the ACP of module 2 to the beginning of the ACP of module 3 was amplified by PCR using as
10 primers the synthetic oligonucleotides:

5'-TACCTGAGGGACCGGCTAGCGGTCTGCCGCGTG-3' and

5'-ATGCTAGCCGTTGTGCCGGCTCGCCGGTCGGTCC-3' and plasmid
pBAM25 as template. The PCR product was treated with T4
15 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid
20 content. The desired plasmid pJLK50 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK52

25 Plasmid pJLK50 was digested with *Nhe*I and the 6.1 kbp insert was ligated with plasmid pJLK35 which had been digested with *Nhe*I. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid
30 content. The desired plasmid pJLK52 was identified by its restriction pattern.

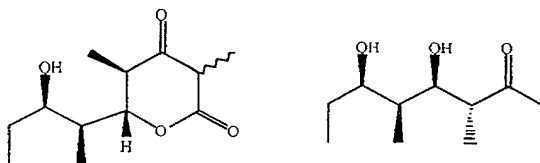
Example 18

- 34 -

Use of plasmid pJLK52 for construction of *S. erythraea* NRRL2338/pJLK52 and the production of tetraketides and macrolides

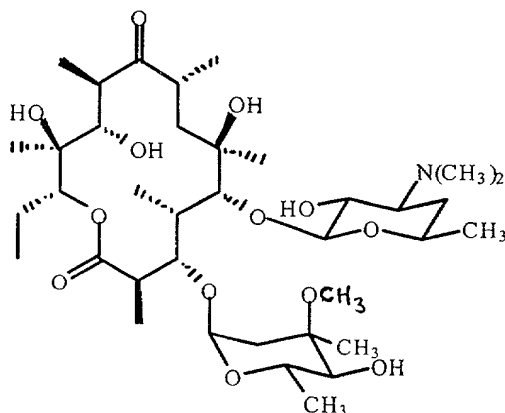
Approximately 5 μ g plasmid pJLK52 were used to transform protoplasts of *S. erythraea* NRRL2338 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA is obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE.

S. erythraea NRRL2338/pJLK52 was used to inoculate SM3 medium containing 5 μ g/ml thiostrepton and allowed to grow for seven to twelve days at 28-30°C. After this time the broth was centrifuged and the pH of the supernatant adjusted to pH=9.5. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. The residue was dissolved in methanol and analysed by GC/MS by HPLC/MS and MS-MS. Tetraketides were identified by GC/MS. The major components were



The following macrolide was identified by HPLC/MS, MS-MS and ¹H-NMR (it was accompanied by products of incomplete processing by post-PKS enzymes)

- 35 -



Example 19

Construction of plasmid pJLK53

Plasmid pJLK53 is a pJLK28 based plasmid containing a PKS gene comprising the ery loading module, the first, the second and the third extension modules of the ery cluster and the ery chain-terminating thioesterase except that the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module has been substituted by the equivalent segment of module 13 of the rapamycin PKS. It was constructed as follows.

Plasmid pJLK50 was digested with NheI and the 6.1 kbp insert was ligated with plasmid pJLK28 which had been digested with NheI. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK53 was identified by its restriction pattern.

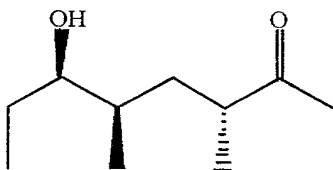
Example 20

- 36 -

Use of plasmid pJLK53 for construction of *S. erythraea* NRRL2338/pJLK53 and the production of TKL derivatives

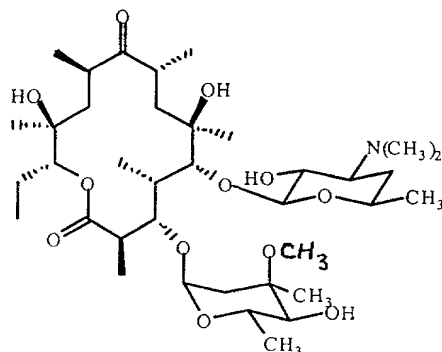
Approximately 5 μ g plasmid pJLK53 were used to transform protoplasts of *S. erythraea* NRRL2338 and stable
5 thiostrepton resistant colonies were isolated. From several colonies total DNA is obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE.

S. erythraea NRRL2338/pJLK53 was used to inoculate SM3
10 medium containing 5 μ g/ml thiostrepton and allowed to grow for seven to ten days at 28-30°C. After this time the broth was centrifuged and the pH of the supernatant adjusted to pH=9.5. The supernatant was then extracted three times with an equal volume of ethyl acetate and the
15 solvent was removed by evaporation. The residue was dissolved in methanol and analysed by GC/MS by HPLC/MS and MS-MS. Tetraketides were identified by GC/MS. The major component was



The following macrolide was identified by HPLC/MS, MS-MS and 1H-NMR (it was accompanied by products of incomplete
30 processing by post-PKS enzymes)

- 37 -



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Example 21

Construction of plasmid pJLK54

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Plasmid pJLK54 is a pJLK29 based plasmid containing a PKS gene comprising the ery loading module, the first, the second and the third extension modules of the ery cluster and the ery chain-terminating thioesterase except that the DNA segment between the end of the acyltransferase and the beginning of the ACP of the second ery extension module has been substituted by the equivalent segment of module 10 of the rapamycin PKS.

It was constructed as follows.

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Plasmid pJLK50 was digested with NheI and the 6.1 kbp insert was ligated with plasmid pJLK29 which had been digested with NheI. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK54 was identified by its restriction pattern.

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Example 22

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Use of plasmid pJLK54 for construction of *S. erythraea* NRRL2338/pJLK54 and the production of tetraketide derivatives and macrolides

- 38 -

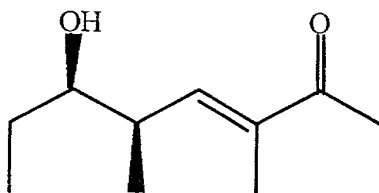
Approximately 5 μ g plasmid pJLK54 were used to transform protoplasts of *S. erythraea* NRRL2338 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA is obtained and analysed by Southern blot hybridisation, to confirm that the plasmid

5 has integrated into the TE.

S. erythraea NRRL2338/pJLK54 was used to inoculate SM3 medium containing 5 μ g/ml thiostrepton and allowed to grow for seven to ten days at 28-30°C. After this time the broth was centrifuged and the pH of the supernatant

10 adjusted to pH=9.5. The supernatant was then extracted three times with an equal volume of ethyl acetate and the solvent was removed by evaporation. The residue was dissolved in methanol and analysed by GC/MS by HPLC/MS and MS-MS. Tetraketides were identified by GC/MS. The major

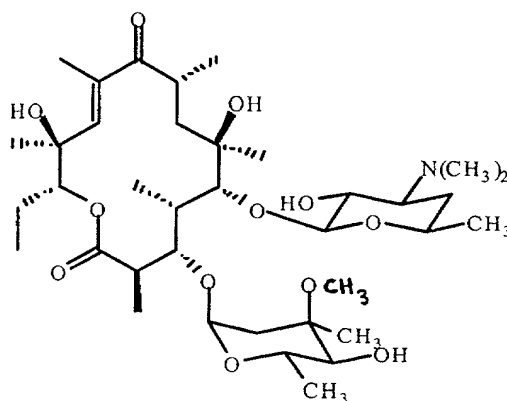
15 component was



25 The following macrolide was identified by HPLC/MS, MS-MS and ¹H-NMR (it was accompanied by products of incomplete processing by post-PKS enzymes)

30

- 39 -



Avermectins

Example 23

Construction of pJLK136

Plasmid pJLK136 is a pWHM3 based plasmid comprising the upstream and the downstream flanking region of the reductive loop of module 2 of the avermectin PKS gene and the erythromycin resistance gene inserted into the mcs which connects these two fragments. Plasmid pWHM3 is described in Vara J et al, J Bacteriol 1989, 171: 5872-5881. Plasmid pJLK136 was constructed via several intermediate plasmids as follows (Figure 6).

Construction of pJLK130

The approximately 2.4 kbp DNA segment of the avermectin PKS gene of *S. avermitilis* encoding the region upstream of the reductive loop of module 2 was amplified by PCR using as primers the synthetic oligonucleotides:

5'-GACGCCGAATTCTTCGGCATCAGCCCCGCGAAG-3' and

5'-

GAGCTAGCAGGTGGGGAGATCTAGGTGGGTGTGGGTGTGGGGTTGGTTGTGGTGGTGG

- 40 -

GTGTA-3' and plasmid pIG22 (Galloway, I. S. (1998) Thesis, University of Cambridge, UK) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK130 was identified by its restriction pattern and DNA sequencing.

10 Construction of pJLK131

The approximately 2.0 kbp DNA segment of the avermectin PKS gene of *S. avermitilis* encoding the region downstream of the reductive loop of module 2 was amplified by PCR using as primers the synthetic oligonucleotides:
5'-GCCCCGGCTAGCCGGCCAGACACACGAACAACAGC-3' and
5'-GGGAATTCCTCGAGGATGACGTGGGCGTTGGTGC-3' and plasmid pIG25 (Galloway, I. S. (1998) Thesis, University of Cambridge, UK) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK131 was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK132

30 Plasmid pJLK130 was digested with NheI and XbaI and the approximately 2.4 kbp insert was ligated with plasmid pJLK131 which had been digested with NheI and XbaI. The ligation mixture was used to transform electrocompetent E.

- 41 -

coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK132 was identified by its restriction pattern.

Construction of plasmid pJLK133

5

Plasmid pJLK117 was digested with BglII and NheI and the approximately 0.1 kbp insert was ligated with plasmid pJLK132 which had been digested with BglII and NheI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK132 was identified by its restriction pattern.

10

Construction of pJLK134

15

The approximately 1.9 kbp DNA segment of the erythromycin gene cluster of *S. erythraea* encoding the erythromycin resistance was amplified by PCR using as primers the synthetic oligonucleotides:

20

5'-TAAGATCTAGCGCTCCGAGGTTCTTGCCCG-3' and

5'-ATGCTAGCCTACCGCTGCCCGGTCCGCCG-3' and plasmid pRH3

(Dhillon, N, et al. Molecular Microbiology (1989) 3:1405-1414) as template. The PCR product was treated with T4

polynucleotide kinase and then ligated with plasmid pUC18,

25

which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture

was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid

content. The desired plasmid pJLK134 was identified by its

30

restriction pattern and DNA sequencing.

Construction of plasmid pJLK135

- 42 -

Plasmid pJLK134 was digested with BglII and NheI and the approximately 1.9 kbp insert was ligated with plasmid pJLK133 which had been digested with BglII and NheI. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK135 was identified by its restriction pattern.

Construction of plasmid pJLK136

Plasmid pJLK135 was digested with EcoRI and the approximately 6.3 kbp insert was ligated with plasmid pWHM3 which had been digested with EcoRI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK136 was identified by its restriction pattern.

Example 24

20

Use of plasmid pJLK136

Approximately 10 μ g plasmid pJLK136 were used to transform protoplasts of *S. avermitilis* (MacNeil, D.J. and Klapko, C.M. Journal of Industrial Microbiology (1987) 2:209-218) and stable thiostrepton and erythromycin resistant colonies were isolated. Individual colonies were selected and subcultured four times in non-selective liquid medium followed by preparation and regeneration of protoplasts (media according to MacNeil T. et al J. Bacteriol. (1993) 175:2552-2563) Thiostrepton sensitive and erythromycin resistant colonies were isolated and characterised by Southern blot hybridisation. One such colony was

- 43 -

designated *S. avermitilis*/JLK1.

Example 25

Construction of plasmid pJLK137

5

Plasmid pJLK120 was digested with BglII and NsiI and the approximately 3.2 kbp insert was ligated with plasmid pJLK133 which had been digested with BglII and NsiI. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK137 was identified by its restriction pattern.

10

Construction of plasmid pJLK138

15

Plasmid pJLK137 was digested with EcoRI and the approximately 7.6 kbp insert was ligated with plasmid pWHM3 which had been digested with EcoRI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK138 was identified by its restriction pattern.

20

25 Example 26

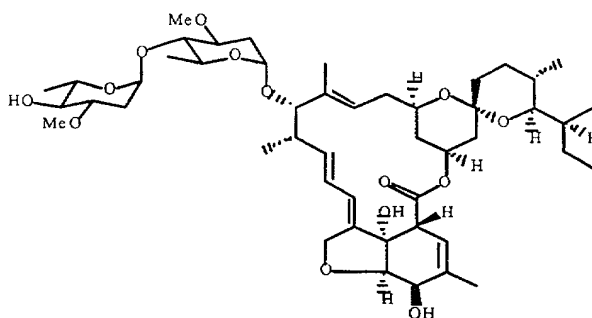
Use of plasmid pJLK138

30

Approximately 10 μ g plasmid pJLK138 were used to transform protoplasts of *S. avermitilis* (MacNeil, D.J. and Klapko, C.M. Journal of Industrial Microbiology (1987) 2:209-218) and stable thiostrepton and erythromycin resistant colonies were isolated. Individual colonies were selected

- 44 -

and subcultured four times in non-selective liquid medium followed by preparation and regeneration of protoplasts (media according to MacNeil T. et al J. Bacteriol. (1993) 175:2552-2563) Thiostrepton and erythromycin sensitive colonies were isolated and characterised by Southern blot hybridisation. One colony of *S. avermitilis*/pJLK138 was used to inoculate liquid media (fermentation according to Pang, C-H. et al J. of Antibiotics (1995) 48:59-66). the cultures were harvested and the products isolated and purified as described in the literature (Pang, C-H. et al J. of Antibiotics (1995) 48:59-66). The products were analysed by HPLC/MS and ¹H-NMR and the following compound could be identified:



Example 27

Construction of plasmid pJLK139

Plasmid pJLK121.1 was digested with BglII and NheI and the 2.2 kbp fragment was ligated with plasmid pJLK133 which had been digested with BglII and NheI. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK139 was

- 45 -

identified by its restriction pattern.

Construction of plasmid pJLK140

Plasmid pJLK139 was digested with EcoRI and the
5 approximately 6.6 kbp insert was ligated with plasmid
pWHM3 which had been digested with EcoRI and then treated
with alkaline phosphatase. The ligation mixture was used
to transform electrocompetent *E. coli* DH10B cells and
individual colonies were checked for their plasmid
10 content. The desired plasmid pJLK140 was identified by its
restriction pattern.

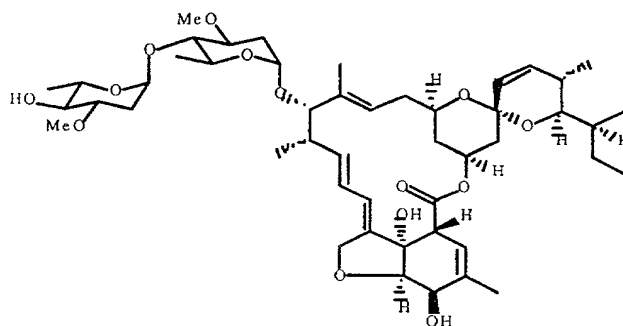
Example 28

15 Use of plasmid pJLK140

Approximately 10 μ g plasmid pJLK140 were used to transform
protoplasts of *S. avermitilis* (MacNeil, D.J. and Klapko,
C.M. Journal of Industrial Microbiology (1987) 2:209-218)
20 and stable thiostrepton and erythromycin resistant
colonies were isolated. Individual colonies were selected
and subcultured four times in non-selective liquid medium
followed by preparation and regeneration of protoplasts
(media according to MacNeil T. et al J. Bacteriol. (1993)
25 175:2552-2563) Thiostrepton and erythromycin sensitive
colonies were isolated and characterised by Southern blot
hybridisation. One colony of *S. avermitilis*/pJLK140 was
used to inoculate liquid media (fermentation according to
Pang, C-H. et al J. of Antibiotics (1995) 48:59-66). the
30 cultures were harvested and the products isolated and
purified as described in the literature (Pang, C-H. et al
J. of Antibiotics (1995) 48:59-66). The products were
analysed by HPLC/MS and ¹H-NMR and the following compound

- 46 -

could be identified:



Example 29

Construction of plasmid pJLK30

pJLK30 is a pJLK117 based plasmid except that the DNA encoding the reductive loop of module 1 of the avermectin PKS has been inserted into the polylinker using the restriction sites BglII and NheI. It was constructed via several intermediate plasmids.

Construction of plasmid pIG67

The approximately 1.7 kbp DNA segment of the gene of the avermectin PKS of *S. avermitilis* encoding the reductive loop of module 1 was amplified by PCR using the following synthetic oligonucleotides as primers:

5'-CCTAGATCCGCCCACCTACCCCTTCCAACACCAG-3' and

5'-TGGGCTAGCGTTTGTGCAACTCCGCCGGTGGAGTG-3' and as template either plasmid pIG155, which contains the first two modules of the avermectin PKS cloned into plasmid pT7-7,

- 47 -

or chromosomal DNA of *Streptomyces avermitilis*. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pIG67 was identified by its restriction pattern and by DNA sequencing.

10 Construction of plasmid pJLK30

Plasmid pIG67 was digested with *Bgl*II and *Nhe*I and the 1.7 kbp fragment was ligated with plasmid pJLK117 which had been digested with *Bgl*II and *Nhe*I. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK30 was identified by its restriction pattern.

20 Example 30

Use of plasmid pJLK30 for the construction of *S. erythraea* JC2/pJLK30 and the production of triketides.

25 Approximately 5 mg of plasmid pJLK30 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation to confirm that the plasmid had integrated into the TE. *S. erythraea* JC2/pJLK30 was
30 plated onto SM3 agar containing 50 mg/ml thiostrepton and

- 48 -

allowed to grow for twelve days at 30°C. 1cm² of the plate was homogenized and extracted with a mixture of 1.2 ml ethyl acetate with 20 ml formic acid. The solvent was decanted and evaporated. The residue was dissolved in methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified as (2R, 3R, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-hexanoic acid δ -lactone and as (2R, 3R, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-heptanoic acid δ -lactone (total of 25 mg/l). Almost none of the corresponding 3-ketolactone could be detected.

Example 31

Construction of plasmid pGMS2

pGMS2 is a pJLK117 based plasmid except that the DNA encoding the reductive loop of module 1 of the avermectin PKS has been inserted into the polylinker using the restriction sites PstI and Bsu36I. It was constructed via several intermediate plasmids.

Construction of plasmid pIG68

The approximately 1.7 kbp DNA segment of the gene of the avermectin PKS of *S. avermitilis* encoding the reductive loop of module 1 was amplified by PCR using the following synthetic oligonucleotides as primers:

5'-TGGCTGCAGAGCTCACAGCCGGGTGCCGGATCCGGTT-3' and

5'-TTTCCTCAGGTCCGCCGGTGGAGTGGGGCGCTGGAC-3' and as template

either plasmid pIG155, which contains the first two modules of the avermectin PKS cloned into plasmid pT7-7,

- 49 -

or chromosomal DNA of *Streptomyces avermitilis*. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pIG68 was identified by its restriction pattern and by DNA sequencing.

10 Construction of plasmid pGMS1

Plasmid pIG68 was digested with *Pst*I and *Bsu*36I and the 1.7 kbp fragment was ligated with plasmid pJLK116 which had been digested with *Pst*I and *Bsu*36I. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pGMS1 was identified by its restriction pattern.

20 Construction of plasmid pGMS2

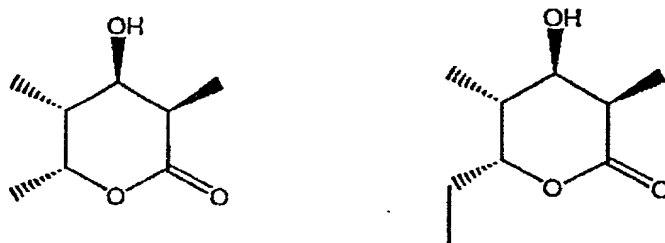
Plasmid pGMS1 was digested with *Nde*I and *Xba*I and the approximately 11.5 kbp fragment was ligated with plasmid pCJR24 which had been digested with *Nde*I and *Xba*I. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pGMS2 was identified by its restriction pattern.

30 Example 32

- 50 -

Use of plasmid pGMS2 for the construction of *S. erythraea* JC2/pGMS2 and the production of triketides.

Approximately 5mg of plasmid pGMS2 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation to confirm that the plasmid had integrated into the TE. *S. erythraea* JC2/pGMS2 was plated onto SM3 agar containing 50µg/ml thiostrepton and allowed to grow for twelve days at 30°C. 1cm² of the plate was homogenized and extracted with a mixture of 1.2 ml ethyl acetate with 20 ml formic acid. The solvent was decanted and evaporated. The residue was dissolved in methanol and analysed by GC/MS and electrospray mass spectroscopy. The products were identified as (2R, 3R, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-hexanoic acid δ-lactone and as (2R, 3R, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-heptanoic acid δ-lactone (total of 17 mg/l), and also a substantial amount of the corresponding 3-ketolactone (5.5 mg/l).



Example 33

- 51 -

Construction of plasmid pJLK31

pJLK31 is a pJLK117 based plasmid except that the DNA encoding the reductive loop of module 2 of the avermectin PKS has been inserted into the polylinker using the restriction sites BglIII and NheI. It was constructed via several intermediate plasmids.

Construction of plasmid pIG69

The approximately 2.4 kbp DNA segment of the gene of the avermectin PKS of *S. avermitilis* encoding the reductive loop of module 2 was amplified by PCR using the following synthetic oligonucleotides as primers:

5'-CCTAGATCTCCCCACCTACCCCTTCCAACACCACCACTACTG-3' and 5'-CCGGCTAGCCGGGCGTGCAGCTGGGCGCCGTTGTCCGCAC-3' and as template either plasmid pIG155, which contains the first two modules of the avermectin PKS cloned into plasmid pT7-7, or chromosomal DNA of *Streptomyces avermitilis*. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pIG69 was identified by its restriction pattern and by DNA sequencing.

Construction of plasmid pJLK31

Plasmid pIG69 was digested with BglIII, NheI and DraI and

- 52 -

the 2.4 kbp fragment was ligated with plasmid pJLK117 which had been digested with BglII and NheI. The ligation mixture was used to transform electrocompetent E.coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK31 was identified
5 by its restriction pattern.

Example 34

Use of plasmid pJLK31 for the construction of *S.*
10 *erythraea* JC2/pJLK31 and the production of triketides.

Approximately 5 mg of plasmid pJLK31 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From
15 several colonies total DNA was obtained and analysed by Southern blot hybridisation to confirm that the plasmid had integrated into the TE. *S. erythraea* JC2/pJLK31 was plated onto SM3 agar containing 50 mg/ml thiostrepton and allowed to grow for twelve days at 30°C. 1cm² of the plate
20 was homogenized and extracted with a mixture of 1.2 ml ethyl acetate with 20 ml formic acid. The solvent was decanted and evaporated. The residue was dissolved in methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified as (2R,
25 3R, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-hexanoic acid δ -lactone and as (2R, 3R, 4S, 5R)-5,3-dihydroxy-2,4-dimethyl-n-heptanoic acid δ -lactone (total of 30 mg/litre).

- 53 -



Example 35

Construction of plasmid pGMS4

pGMS4 is a pJLK117 based plasmid except that the DNA encoding the reductive loop of module 2 of the avermectin PKS has been inserted into the polylinker using the restriction sites *Sna*BI and *Bsu*36I. It was constructed via several intermediate plasmids.

Construction of plasmid pIG70

The approximately 2.4 kbp DNA segment of the gene of the avermectin PKS of *S. avermitilis* encoding the reductive loop of module 2 was amplified by PCR using the following synthetic oligonucleotides as primers:

5'-CCCTACGTACCCCTTCCAACACCACTACTGGCTCGAAAG-3' and

5'-GGCCCTCAGGTGGGCGCCGTTGTCCGCACCACCGGTA-3' as template

either plasmid pIG155, which contains the first two modules of the avermectin PKS cloned into plasmid pT7-7, or chromosomal DNA of *Streptomyces avermitilis*. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by

- 54 -

digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E.coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pIG70 was identified by its restriction pattern and by DNA sequencing.

Construction of plasmid pGMS3

Plasmid pIG70 was digested with SnaBI, Bsu36I and DraI and the 2.4 kbp fragment was ligated with plasmid pJLK116 which had been digested with SnaBI and Bsu36I. The ligation mixture was used to transform electrocompetent E.coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pGMS3 was identified by its restriction pattern.

Construction of plasmid pGMS4

Plasmid pGMS2 was digested with NdeI and XbaI and the approximately 12.4 kbp fragment was ligated with plasmid pCJR24 which had been digested with NdeI and XbaI. The ligation mixture was used to transform electrocompetent E.coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pGMS4 was identified by its restriction pattern.

Example 36

Use of plasmid pGMS4 for the construction of *S. erythraea* JC2/pGMS4 and the production of triketides.

- 55 -

Approximately 5 mg of plasmid pGMS4 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation to confirm that the plasmid had integrated into the TE. *S. erythraea* JC2/pGMS4 was plated onto SM3 agar containing 50 mg/ml thiostrepton and allowed to grow for twelve days at 30°C. 1cm² of the plate was homogenized and extracted with a mixture of 1.2 ml ethyl acetate with 20 ml formic acid. The solvent was decanted and evaporated. The residue was dissolved in methanol and analysed by GC/MS and electrospray mass spectroscopy. Only traces of putative triketide products were detected.

Example 37

Construction of plasmid pJLK27

Plasmid pJLK27 is a pJLK114 based plasmid except that the DNA fragment encoding the reductive loop of module 13 of the rap PKS has been inserted into the mcs. It was constructed via several intermediate plasmids as follows.

Construction of plasmid pJLK120a

The approximately 3.2 kbp DNA segment of the rapC gene of *S. hygrosopicus* encoding the reductive loop of module 13 was amplified by PCR using as primers the synthetic oligonucleotides:

5'-TACCTAGGCACCACCACAACCCGGGTA-3' and

5'-TACAATTGGCCCGCGAGTCCCCGACGCT-3' and cosmid cos 31

(Schwecke, T. et al. (1995) Proc. Natl. Acad. Sci. USA

- 56 -

92:7839-7843) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK120a was identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK27

Plasmid pJLK120a was digested with AvrII and HpaI and the 3.2 kbp fragment was ligated with plasmid pJLK114 which had been digested with AvrII and HpaI. The ligation mixture was used to transform electrocompetent E. coli DH10B cells and individual colonies were checked for their plasmid content. The desired plasmid pJLK27 was identified by its restriction pattern.

Example 38

Use of plasmid pJLK27 for construction of JC2/pJLK27 and the production of triketides

Approximately 5 mg plasmid pJLK27 were used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies were isolated. From several colonies total DNA was obtained and analysed by Southern blot hybridisation, to confirm that the plasmid has integrated into the TE. JC2/pJLK27 was plated onto SM3 agar containing 50 mg/ml thiostrepton and allowed to grow for twelve days at 30°C. 1 cm² (0.5 ml) of the plate was

- 57 -

homogenised and extracted with a mixture of 1.2 ml ethyl acetate and 20 ml formic acid. The solvent was decanted and removed by evaporation and the residue dissolved in methanol and analysed by GC/MS and electrospray mass spectroscopy. The major products were identified (by comparison with authentic material) as (2R, 4S, 5R)-2,4-dimethyl-5-hydroxy-n-hexanoic acid δ -lactone and as (2R, 4S, 5R)-2,4-dimethyl-5-hydroxy-n-heptanoic acid δ -lactone (total of 41 mg/l), with some of the corresponding 3-ketolactones (total of 12 mg/l) and 3-hydroxylactones (total of 2.8 mg).



All documents and sequence deposits referred to herein are explicitly and individually incorporated herein by reference.

- 58 -

CLAIMS

1. A nucleic acid molecule encoding at least part of a Type I polyketide synthase, said part comprising at least part of an extension module, wherein the nucleic acid
5 has, in place of one or more genes encoding enzymes associated with reduction, a polylinker with multiple restriction enzyme sites.

2. A nucleic acid according to claim 1 wherein the polylinker is in place of all genes encoding enzymes
10 which are associated with reduction and which are normally included in said extension module.

3. A nucleic acid encoding at least part of a Type I polyketide synthase, said part comprising at least part
15 of an extension module, wherein the nucleic acid has a polylinker with multiple restriction enzyme sites, which polylinker connects nucleic acid encoding at least part of an acyl transferase enzyme to nucleic acid encoding at
20 least part of an acyl carrier protein.

4. A nucleic acid according to any preceding claim wherein at least some of the restriction sites included
25 in the polylinker are absent from the Type I polyketide synthase-encoding nucleic acid.

5. A nucleic acid according to any preceding claim wherein at least some of the restriction sites included
30 in the polylinker are uncommon in or absent from other naturally occurring nucleic acid sequences which encode reductive enzymes of Type I polyketide synthases.

6. A nucleic acid according to any preceding claim

- 59 -

wherein the polylinker includes at least some of the following restriction sites: AvrII, BglII; SnaBI; PstI; SpeI; NsiI; Bsu36I; NheI; and HpaI.

7. A nucleic acid according to any preceding claim
5 which additionally encodes a loading module

8. A nucleic acid according to any preceding claim
10 which additionally encodes one or more further extension modules.

9. A nucleic acid according to any preceding claim
15 further including a nucleic acid sequence incorporated into the polylinker, which incorporated nucleic acid encodes one or more reductive enzymes.

10. A nucleic acid according to claim 9 wherein said one
or more reductive enzymes is/are a β -ketoreductase, a
dehydratase and/or an enoyl reductase.

11. A nucleic acid according to claim 10 wherein said
20 one or more reductive enzymes include(s) at least a β -ketoreductase.

12. A nucleic acid according to claim 10 or claim 11
25 wherein at least one of said one or more reductive enzymes is from a different extension module of the same polyketide synthase as said at least part of a Type I polyketide synthase.

13. A nucleic acid according to any one of claims 10 to
30 12 wherein at least one of said one or more reductive enzymes is from a different polyketide synthase.

- 60 -

14. A vector including a nucleic acid as defined in any preceding claim.

15. A host cell transfected, transformed or conjugated with a nucleic acid or vector as defined in any preceding claim.

16. A host cell according to claim 15 which is a cell of a *Streptomyces* species.

17. A host cell according to claim 16 which is a cell of *S. erythraea* or *S. avermitilis*.

18. A method for producing a nucleic acid encoding a novel polyketide synthase, the method including the steps of:

i. providing a nucleic acid as defined in any one of claims 1 to 8; and

ii. incorporating into said nucleic acid a nucleic acid sequence which encodes at least one reductive enzyme.

19. A method according to claim 18 wherein said nucleic acid sequence encoding at least one reductive enzyme is as defined in any one of claims 9 to 13.

20. A method for producing a fermentation product containing a polyketide, the method including the step of culturing a host cell as defined in claim 15.

21. A fermentation product containing a C22-C23 dihydroavermectin, substantially free of other macrolides.

- 61 -

22. A fermentation product according to claim 21 wherein the dihydroavermectin is ivermectin.

23. A fermentation product containing a B1 avermectin substantially free of B2 avermectins.

5

24. A method for producing a polyketide, the method including the steps of:

i. providing a fermentation product resulting from the method of claim 20, or a fermentation product according to any of claims 21-23; and

10

ii. at least partially purifying a polyketide from said fermentation product.

25. A method according to claim 24 wherein the polyketide is an avermectin.

15

26. A method according to claim 25 wherein the avermectin is a B1 avermectin.

27. A method according to claim 25 wherein the avermectin is a B1 avermectin.

20

Fig. 1

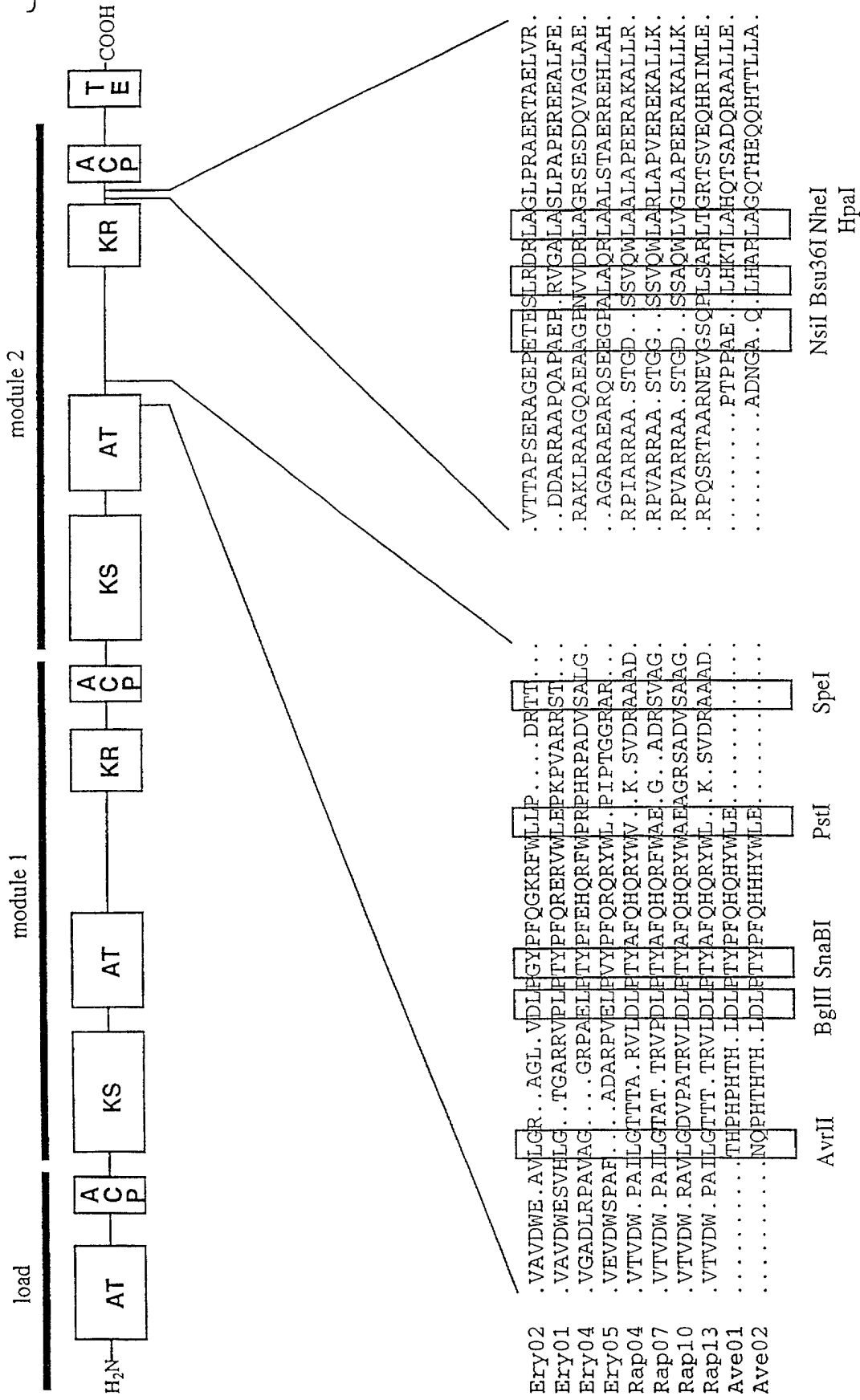
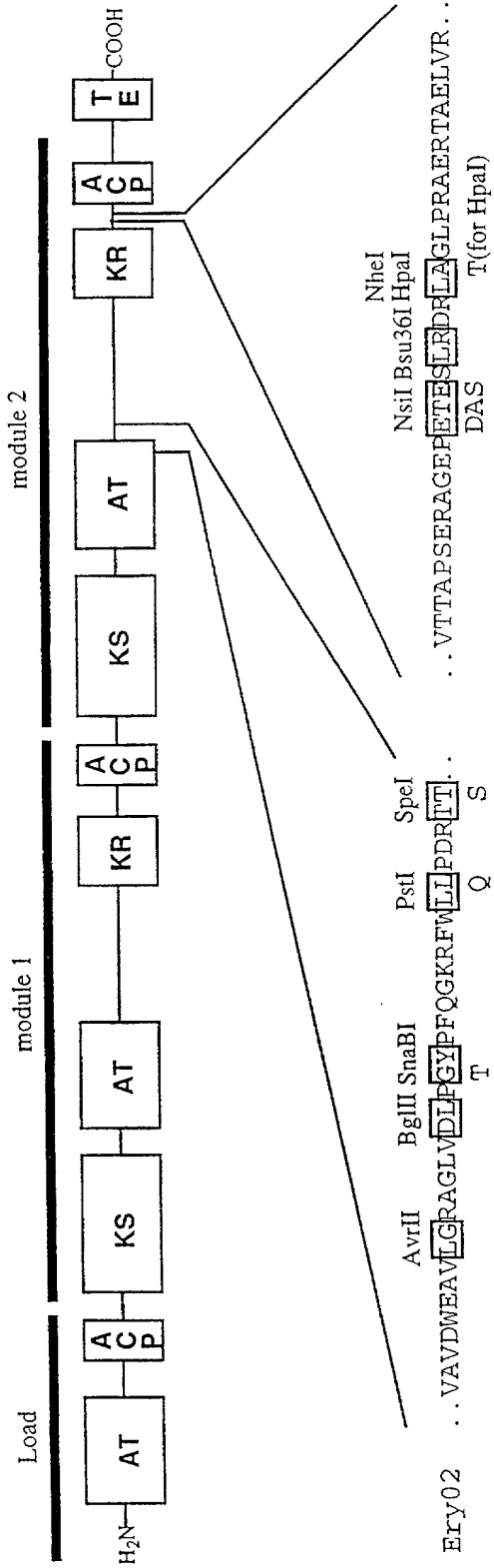


Fig. 2



The cloning strategy for pJLK117 involves the following steps:

- Three plasmids are used as starting materials: pJLK01 (PstI, Scal, AvrII), pJLK02 (PstI, AvrII, HpaI), and pJLK03 (PstI, HpaI, XbaI).
- pJLK01 and pJLK02 are digested with PstI/AvrII to create pJLK05.
- pJLK02 and pJLK03 are digested with PstI/HpaI to create pJLK04.
- pJLK05 and pJLK04 are digested with Scal/XbaI to create pJLK07.
- pJLK07 and pJLK04 are digested with NdeI/Scal to create pJLK114.
- pJLK114 and pJLK04 are digested with AvrII/HpaI to create pJLK115.
- pJLK115 and pJLK04 are digested with NdeI/XbaI to create pJLK116.
- pJLK116 and pJLK04 are digested with Bsu36I/XbaI to create pJLK117.

The final plasmid pJLK117 contains the mcs-fragment, the pJLK01 fragment, and the pJLK03 fragment.

Fig. 4

forward (Plf):

5'-CTA GGC CGG GCC GGA CTG GTA GAT CTG CCT ACG TAT CCT TTC CAG
 GGC AAG CGG TTC TGG CTG CAG CCG GAC CGC ACT AGT CCT CGT GAC GAG
 GGA GAT GCA TCG AGC CTG AGG GAC CGG TT-3'

backward (Plb):

5'-AAC CGG TCC CTC AGG CTC GAT GCA TCT CCC TCG TCA CGA GGA CTA GTG
 CGG TCC GGC TGC AGC CAG AAC CGC TTG CCC TGG AAA GGA TAC GTA
 GGC AGA TCT ACC AGT CCG GCC CGG C-3'

oligos annealed:

CTAGGCCGGGCCGACTGGTAGATCTGCCTACGTATCCTTTCCAGGGCAAGCGTTCTGGCTGCAG...
 CGGCCCCGGCCTGACCATCTAGACGGATGCATAGGAAAGGTCCCCTTCGCCAAGACCGACGTC...

 AvrII BglII SnaBI PstI

... CCGGACCGCACTAGTCCTCGTGACGAGGGAGATGCATCGAGCCTGAGGGACCGGTT
 ... GGCCTGGCGTGATCAGGAGCACTGCTCCCTCTACGTAGCTCGGACTCCCTGGCCAA

 SpeI NsiI Bsu36I HpaI

Fig. 5a

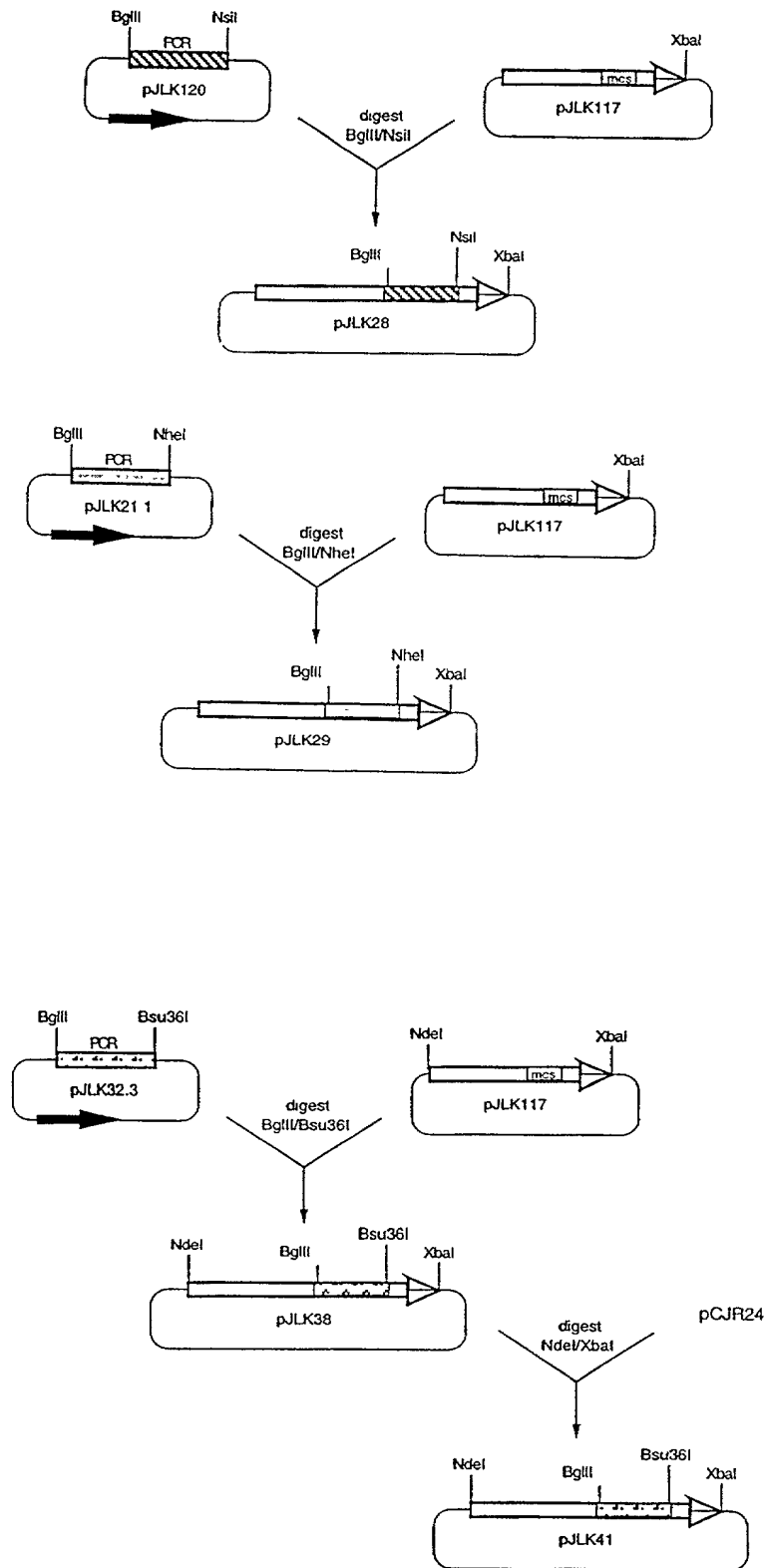


Fig. 5b

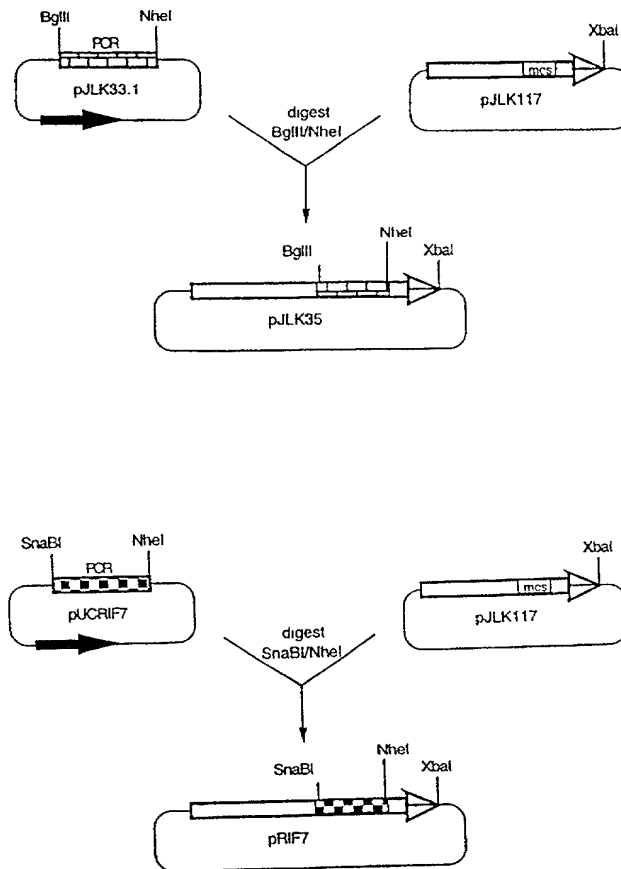


Fig. 6

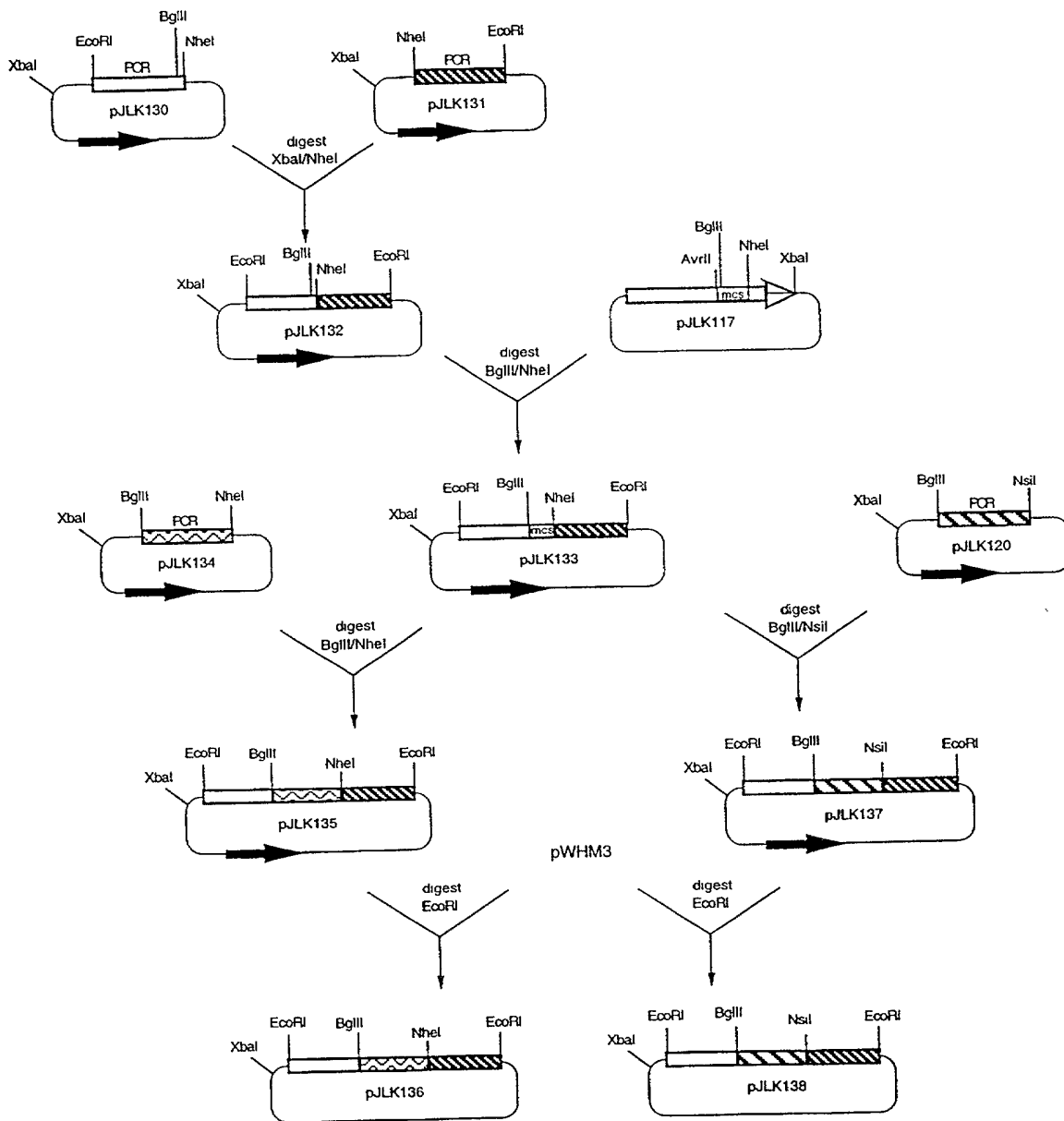


Fig. 7a

1	CCCGGGCGAT	CTCCCGGATC	ACCTGTGCGG	GGCTGGGCAT	GTGCAGGAGA
51	CACTCCAGGG	CCCACGCCGC	GTCGAAGGAC	CCGTCGGGAA	ACGGCAGTTC
101	CATCGCGTCG	GCACGGGTGA	ACACGACCCG	GTCCGCCACG	TGCGACTGCT
151	TCGCGAGAGC	GGTCGCCAGC	CCGACCTGAA	CCTCGCTCAC	CGTCACGCCG
201	ACGACATCGA	CGGGCGCGCT	CAGGGCGAGC	CGCACCGCCG	GCTTTCCGGA
251	ACCGCAGCCG	ACGTCCAGGA	CCCGGCGGCC	CGTGATGCCT	CTCAGCTTGC
301	CGATGAGGAG	ATCGGTGAGC	CGGTGCGCGG	CCTTGCCCCG	TGAACTGCCG
351	TCCCCCGGCT	GCGGCCAGTA	TCCGAGGTGG	GTGTTCCAC	CCAGCGCACG
401	ATTCATGAGG	TCGGTCAAAC	GGTCGTAAGT	GTCCCCCACT	TCCAGGGAAG
451	AGGGCGGGGT	CTGCTCCGGG	ACGGCCATCA	TGGTCGGGAA	CCTCCGCAAT
501	CCGGGCCCGG	CGGCCAGCT	GTCGTGGCGA	TCTACTCCAG	GAAACGTCGA
551	CCTTTTTCTG	CCACTTGTCC	GAGCTATGCA	GACACCCCGA	TCCCCTAAGA
601	AATGAACACC	CTTGGAACG	GCACAGCCCA	GGGGTGGATA	GGGGTATTTC
651	CCGCCGCCGC	GCCGTCATTA	GCTTTGAAGA	GTTGAAGACG	TTCAAGACAT
701	TGATGCCCGG	CCGTCAGCGG	ATTTCTCGCG	CTCCTTTCAT	TCTTCGACGC
751	TGCATTGCAG	CTCTCATCAT	GTCCGCACGG	CCGCCGAGCA	TTGCCTAGCG
801	GTGAGGACAC	AGCTCAGGTG	CAGAGGATGG	ACGGCGGGGA	AGAACCCCGC
851	CCTGCGGCAG	GGGAGGTCCT	CGGAGTGGCC	GACGAGGCGG	ACGGCGGCGT
901	CGTCTTCGTT	TTTCCCGGGC	AGGGCCCGCA	ATGGCCGGGC	ATGGGAAGGG
951	AACTTCTCGA	CGCTTCCGAC	GTCTTCCGGG	AGAGCGTCCG	CGCCTGCGAA
1001	GCCGCGTTCG	CGCCCTACGT	CGACTGGTCG	GTGGAGCAGG	TGTTGCGGGA
1051	CTCGCCGGAC	GCTCCCGGGC	TGGACCGGGT	GGACGTCGTC	CAGCCGACCC
1101	TGTTTCGCCGT	CATGATCTCC	CTGGCCGCC	TCTGGCGCTC	GCAAGGGGTC
1151	GAGCCGTGCG	CGGTGCTGGG	ACACAGCCTG	GGCGAGATCG	CGGCAGCCCA
1201	CGTCTCGGGA	GGCCTGTCCC	TGGCCGACGC	CGCACGCGTG	GTGACGCTTT
1251	GGAGCCAGGC	ACAGACCACC	CTTGCCGGGA	CCGGCGCGCT	CGTCTCCGTC
1301	GCCGCCACGC	CGGATGAGCT	CCTGCCCCGA	ATCGCTCCGT	GGACCGAGGA
1351	CAACCCGGCG	CGGCTCGCCG	TCGCAGCCGT	CAACGGACCC	CGGAGCACAG
1401	TCGTTTCCGG	TGCCCCGCGAG	GCCGTCGCGG	ACCTGGTGGC	CGACCTCACC
1451	GCCGCGCAGG	TGCGCACGCG	CATGATCCCG	GTGGACGTTT	CCGCCCACTC
1501	CCCCCTGATG	TACGCCATCG	AGGAACGGGT	CGTCAGCGGC	CTGCTGCCCA
1551	TCACCCACAG	CCCCTCCCGC	ATCCCCTTCC	ACTCCTCGGT	GACCGGCGGC
1601	CGCCTCGACA	CCCGCGAGCT	AGACGCGGCG	TACTGGTACC	GCAACATGTC
1651	GAGCACGGTC	CGGTTGAGC	CCGCCGCCCG	GCTGCTTCTG	CAGCAGGGGC
1701	CCAAGACGTT	CGTCGAGATG	AGCCCGCACC	CGGTGCTGAC	CATGGGCCTC
1751	CAGGAGCTCG	CCGCGGACCT	GGGCGACACC	ACCGGCACCG	CCGACACCGT
1801	GATCATGGGC	ACGCTGCGCC	GCGGCCAGGG	CACCCTGGAC	CACTTCCTGA
1851	CGTCTCTCGC	CCAACCTACG	GGGCATGGTG	AGACGTCGGC	GACCACCGTC
1901	CTCTCGGCAC	GCCTGACCGC	GCTGTCCCCC	ACGCAGCAGC	AGTCGCTGCT
1951	CCTGGACCTG	GTGCGCGCCC	ACACCATGGC	GGTGCTGAAC	GACGACGGAA
2001	ACGAGCGCAC	CGCGTCGGAT	GCCGGCCCAT	CGGCGAGTTT	CGCCACCTC
2051	GGCTTCGACT	CCGTCATGGG	TGTCGAAGTG	CGCAACCGCC	TCAGCAAGGC
2101	CACGGGCCTG	CGGTTGCCCG	TGACGCTCAT	CTTCGACCAC	ACCACGCCGG
2151	CCGCGGTCGC	CGCGCGCCTT	CGGACCGCGG	CGCTCGGCCA	CCTCGACGAG
2201	GACACCGCGC	CCGTACCGGA	CTACCCAGC	GGCCACGGAG	GCACGGCAGC
2251	GGCGGACGAC	CCGATCGCCA	TCATCGGCAT	GGCATGCCGT	TTCCCGGGCG

Fig. 7b

2301 GAGTCCGGTC CCCGAAGGAC CTGTGGGAGC TGCCCGCCTC GGGCGGAGAC
2351 GCCATCGGGC CGTTCCTCCAC CGACCGCGGA TGGCCACCGG AACAGCGTCA
2401 CGCCCAGGAC CCCACGCAGC CCGGCACGTT CTATCCGCAG GGAGGCGGGT
2451 TCCTTCACGA CGCGGCGCAC TTCGACGCCG GCTTCTTCGG AATCAGTCCA
2501 CGTGAGGCAC TGGCGATGGA TCCGCAGCAG CGGCTGCTGC TGGAGACGTC
2551 CTGGGAGGCG TTCGAGCGGG CGGGAATCGA TCCGCTGTCG GTACGCGGGT
2601 CCCGTACGGG CGTCTTCGCG GGCGCCCTCT CTTTCGACTA CGGCCCGCGT
2651 ATGGACACCG CGTCGTCGGA GGGCGCCGCG GACGTGGAGG GCCACATCCT
2701 CACCGGTACC ACGGGCAGCG TCCTGTTCGGG CCGTATCGCC TACAGCTTCG
2751 GGCTGGAAGG GCCGGCGATC ACCGTGGACA CGGGGTGCTC GGCATCGCTC
2801 GTGACGCTGC ATCTGGCGTG CCAGTCGCTG CGGTGCGGTG AGTGCACGCT
2851 CGCGCTGGCC GGCGGCGTCT CGGTCAATGTC CACCCTCGGC ATGTTTCATCG
2901 AGTTCTCCCG GCAGCGCGGG CTGTGCGGTG ACGGCAGGTG CAAGGCGTAC
2951 TCGGCTGCAG CCGACGGCAC CGGCTGGGGC GAGGGCGTCG GGATGCTGTT
3001 GGTGGAGCGG TTGTGCGATG CGGTGCGGCT GGGGCATCGG GTGCTGGCGG
3051 TGGTACGCGG CAGTGCGGTC AACCAGGACG GTGCGTCGAA TGGGCTGACG
3101 GCGCCGAACG GTCCGGCTCA GGAGCGGGTG ATCCGGCAGG CGTTGGCGAA
3151 CGCGGGGTTG TCCGTGGCGG ATGTGGATGT GGTGGAGGGG CACGGGACGG
3201 GCACGACGCT GGGTGATCCG ATCGAGGCAC AGGCGTTGCT CGCCACGTAC
3251 GGGCAGCGGG CCGGTGACAG GCCGCTGTGG CTGGGGTCTC TGAAGTCCAA
3301 CATCGGGCAC ACCATGGCTG CCGCGGGTGT GGGTGGGGTC ATCAAGATGG
3351 TGATGGCGTT GCGGGAGGGG GTGTTGCCGC GGACGTTGCA TGTGGATGAG
3401 CCGTCGCCGC AGGTGGACTG GTCCGCGGGG GCGGTGCGGC TGCTGACGGA
3451 GGCGGTGCCG TGGCCGGGGG ACCCGGCAGG GCGGTGCGG CGGGCGGGAG
3501 TGTCGTCGTT CGGGATCGGC GGCACGAATG CGCATGTGAT TTTGGAGGAG
3551 GCGCCGGCGG CGGGGGGCTG TGTGCGCGG GGTGGGGTGT TGGAGGGTGC
3601 TCCGGGTCTT GCCATTTCCG TGGCTGAGTC GGTGGCCGCT CCAGTGGCTG
3651 TGTCTGCGCC GGTGGCTGAG TCGGTGCCG TGCCGGTGCC GGTGCCGGTT
3701 CCTGTGCCGG TGTCGGCTAG GTCTGAGGCT GGGTTGCGGG CGCAGGCGGA
3751 GGCGTTGCGT CAGTACGTGG CAGTCCGGCC GGACGTTTCG CTTGCCGATG
3801 TGGGTGCGGG TCTGGCCTGT GGGCGGGCTG TGCTGGAGCA TCGTGCGGTC
3851 GTCCTGGCCG CGGACCGTGA GGAGCTGGTG CAAGGGTTGG GGGCGCTGGC
3901 GGCGGGTGAG CCGGATCGGC GGGTGACCAC GGGTCATGCG CCGGGTGGTG
3951 ACCGGGGCGG TGTCGTCTTC GTGTTTCCCG GACAGGGTGG GCAGTGGGCC
4001 GGGATGGGTG TGCGTCTGCT CGCCTCCTCT CCGGTGTTCTG CCCGGCGGAT
4051 GCAGGCGTGC GAGGAGGCTC TGGCGCCGTG GGTGGACTGG TCTGTGGTGG
4101 ACATCCTGCG CCGGGACGCG GGGGATGCGG TGTGGGAGCG GGCCGATGTG
4151 GTCCAGCCTG TGCTGTTTCA CGTCATGGTG TCTTTGGCTG CTCTGTGGCG
4201 TTCCTACGGT ATCGAACCCG ACGCGGTCCT TGGCCATTCC CAGGGCGAGA
4251 TCGCGGCCGC GCATGTGTGT GGGGCGCTGA GCCTGAAGGA CGCGGCGAAG
4301 ACTGTTGCGC TGCGCAGCCG GCGGCTGGCC GCTGTGCGGG GCCGGGGCGG
4351 CATGGCCTCA GTGCCGCTGC CTGCCCAGGA GGTGGAGCAG CTCATTGGTG
4401 AGCGGTGGGC GGGGCGGTTG TGGGTGGCGG CGGTCAACGG CCCCCTCTCC
4451 ACCGCCGTCT CGGGGGATGC CGAGGCGGTG GACGAGGTGC TGGCGTACTG
4501 TGCCGGCACC GGGGTGCGGG CCCGGCGGAT CCCGGTCGAC TATGCCTCGC
4551 ACTGCCCCCA TGTGCAGCCC CTGCGGGAGG AGTTGCTGGA GCTGCTGGGG

Fig. 7c

4601 GACATCAGCC CGCAGCCGTC CGGCGTGCCG TTCTTCTCCA CGGTGGAGGG
4651 CACCTGGCTG GACACCACAA CCCTGGACGC CGCCTACTGG TACCGCAACC
4701 TGCACCAGCC GGTCCGTTTC AGCGATGCCG TCCAGGCCCT GGCGGATGAC
4751 GGACACCGCG TCTTCGTGCA AGTCAGCCCC CACCCACCC TCGTCCCCGC
4801 CATCGAAGAC ACCACCGAAG ACACCGCCGA AGACGTCACC GCGATCGGCA
4851 GCCTCCGCCG CGGCGACAAC GACACCCGCC GCTTCCTCAC CGCCCTCGCC
4901 CACACCCATA CCACCGGCAT CGGCACACCC ACCACCTGGC ACCACCACTA
4951 CACCCACCAC CACACCCACC CCCACCCCA CACGCACCTC GACCTGCCCA
5001 CCTACCCCTT CCAACACCAG CACTACTGGC TCGAGAGCTC ACAGCCGGGT
5051 GCCGGATCCG GTTCGGGTGC CGGTGCCGGT TCGGGTGCCG GTTCCGGGCG
5101 GGCAGGGACT GCGGGCGGGA CGGCAGAGGT GGAGTCGCGG TTCTGGGACG
5151 CGGTGGCCCG CCAGGACCTG GAAACGGTCG CGACCACACT CGCCGTGCCC
5201 CCCTCCGCCG GCCTGGACAC GGTGGTGCCC GCACTCTCCG CCTGGCACCG
5251 CCACCAACAC GACCAAGCCC GCATCAACAC CTGGACCTAC CAGGAAACCT
5301 GGAAACCCCT CACCCTCCCC ACCACCCACC AACCCACCA AACCTGGCTC
5351 ATCGCCATCC CCGAAACCCA GACCCACCAC CCCCACATCA CCAACATCCT
5401 CACCAACCTC CACCACCACG GCATCACCCC CATCCCCCTC ACCCTCAACC
5451 ACACCCACAC CAACCCCCAA CACCTCCACC ACACCCTCCA CCACACCCGA
5501 CAACAAGCCC AAAACCACAC CACCGGAGCC ATCACCGGCC TGCTCTCCCT
5551 CCTCGCCCTC GACGAAACAC CCCACCCCA CCACCCCCAC ACACCCACCG
5601 GCACCTCCT CAACCTCACC CTCACCCAAA CCCACACCCA AACCCACCCA
5651 CCAACCCCCC TCTGGTACGC CACCACCAAC GCCACCACCA CCCACCCCAA
5701 CGACCCCTC ACACACCCCA CCCAAGCCCA AACCTGGGGA CTCGCCCGCA
5751 CCACCTCCT CGAACACCCC ACCCACACCG CCGGAATCAT CGACCTCCCC
5801 ACCACCCCA CCCCCACAC CCTCCACCAC CTCACCCAAA CCCTCACCCA
5851 ACCCCACCAC CAAACCCAAC TCGCCATCCG CACCACCGGC ACCCACACCC
5901 GCCGCCCTAC CCCCACCACC CTCACCCCA CACACCAACC ACCCACCCCC
5951 ACCCCCCACG GAACCACCT CATCACCGGC GGAACCGGCG CCCTCGCCAC
6001 CCACCTCACC CACCACCTCA CCACCCACCA ACCCACCAA CACCTCCTCC
6051 TCACCAGCCG AACC GGCCCC CACACCCCC ACGCACAACA CCTCACCACC
6101 CAACTCCAAC AAAAAGGCAT CCACCTCACC ATCACCACCT GCGACACCAG
6151 CAACCCAGAC CAACTCCAAC ATCTCCTCAA CACCATCCCC CCACAACACC
6201 CCCTCACCAC CGTCATCCAC ACCGCAGGCA TCCTCGACGA CGCCACCCTC
6251 ACCAACCTCA CCCCCACCCA ACTCAACAAC GTCCTCCGCG CCAAAGCCCA
6301 CAGCGCCAC CTCCTCCACC AACTCACCCA ACACACCCCT CTCACCGCCT
6351 TCGTCTCTA CTCCTCCGCC GCCGCCACCT TCGGCGCACC CGGCCAAGCC
6401 AACTACGCCG CAGCCAACGC CTACCTCGAC GCCCTCGCCC ACCACCGCCA
6451 CACCCACCAC CTCCCCGCCA CCAGCATCGC CTGGGGCACC TGGCAAGGAA
6501 ACGGACTCGC TGATTGCGAC AAGGCCCGCG CATATCTCGA CCGCCGCGGG
6551 TTTCGACCA TGTCACCCGA GTTGCCACG GCAGCGGTCA CGCAGGCGAT
6601 CGCGGACACC GAACGGCCGT ATGTCGTCAT CGCCGACATC GACTGGAGCA
6651 AGATCGAACA CACCTCTCAG ACCAGCGACC TGGTGAGCGC GGCCCGGGAA
6701 AGGGAGCCAG CTGTCCAGCG CCCACTCCA CCGGCGGAGT TGCACAAAAC
6751 GCTGGCCCAT CAGACGTCGG CCGACCAACG GGCCGCATTG CTCGAGCTCG
6801 TACGAGACCA TGTGGCGGCA GTGCTCCGGC ACGCGGACCC GAAAGCCATC
6851 GCGCCCGACC AGTCGTTCCG TGCACCTCGC TTCGATTAC TCACGGCCGT

Fig. 7d

6901	CGAGTTCCGA	AACCTGCTGA	TCAAGGCAAC	AGGACTCCGC	CTTCCTGTCT
6951	CGCTGGTCTT	CGACCACCCG	ACCCCTGCCA	AACTCGCCGT	ACACCTGCAG
7001	AACCAACTGC	GGGGCACAGC	AGCGGAGTCG	GCTCCTTCAG	CGGCAGCCGT
7051	TACCGCCGAG	GCTTCTGTCA	CCGAGCCGAT	CGCCATCGTT	GGCATGGCCT
7101	GTCGTTTCCC	CGGCGGAGTG	ACCTCGGCGG	ACGACTTCTG	GGATCTGATC
7151	TCCTCCGAGC	AGGACGCGAT	CGGCGGATTC	CCCACCGACC	GCGGCTGGGA
7201	CCTGGACACG	CTCTACGACC	CCGACCCCGA	CCACCCCGGC	ACCTGCTACA
7251	CCCGAAACGG	CGGATTCTTC	TACGACGCAG	GCCACTTCGA	CGCCGAATTC
7301	TTGCGCATCA	GCCCCCGCGA	AGCCCTCGCC	ATGGACCCCC	AGCAACGACT
7351	CCTCCTCGAA	ACCGCCTGGG	AAACCATCGA	ACACGCCGGC	ATCAACCCCC
7401	ACACCCTCCA	CGGCACCCCC	ACCGGAGTCT	TCACCGGCAC	CAACGGACAG
7451	GACTACGCAC	TTGCGGTGCA	CAACGCGGGC	CAGTCAACCG	ATGGTTTTCGC
7501	ACTGACCGGA	ACCGCCGGCA	GCGTCATCTC	CGGTCGTATC	TCGTACACGT
7551	TTGGTTTTGA	GGTCTCTGCG	GTGTCGGTGG	ACACGGCTTG	TTCTCTGTCG
7601	TTGGTGGCTT	TGCATCTGGC	CTGTCAAGCG	TTGCGTGCGG	GTGAGTGCTC
7651	GATGGCGCTT	GCCGGGGGTG	TGACGGTGAT	GTCGTCTCCG	GGTGCCTTCG
7701	TGGAGTTTTT	GCGGCAGCGG	GGTCTGGCCG	CGGACGGGCA	TTGCAAGGCG
7751	TTCTCGGCGG	CGGCGGACGG	GACCGGCTGG	GGTGAGGGTG	TGGGGATGCT
7801	GCTGGTGGAG	CGGCTCTCCG	ACGCCCATCG	CAACGGTCAC	CGTGTCTTGG
7851	CCGTGGTGGC	TGGCAGTGCG	GTCAACCAGG	ACGGTGCGAG	CAACGGTCTG
7901	ACCGCGCCCA	ACGGGCCGTC	CCAGCAGCGT	GTCATCCGCC	AGGCCCTCGC
7951	CAACGCCGGC	TTGTCTGGCC	GTGATGTCGA	CGCGGTGGAG	GCCACGGCA
8001	CCGGCACCAC	TTTGGGCGAC	CCGATCGAGG	CCCAGGCCCT	CCTCGCGACC
8051	TACGGACAGG	ACCGTGCCGG	CGAGGGGCCG	CTGTGGCTGG	GCTCGGTCAA
8101	GTCCAATGTC	GGTCACACAC	AGGCTGCCGC	GGGCGTCCGC	GGGGTGATCA
8151	AGATGGTGAT	GGCGCTGCGG	CATGGTCTGC	TGCCGCGGAC	GTTGCATGTG
8201	GATGAGCCGT	CGCCGCATGT	GGA CTGGTCC	GCGGGTGCGG	TGCAGCTGCT
8251	GACGGAGACG	GTGCCCTGGC	CCGGCGGGGA	GGGGCGGCTA	CGGCGGGCAG
8301	GAGTGTCATC	ATTGCGCGTC	AGCGGCACCA	ACGCCACAGT	CATCCTCGAA
8351	GAAGCACCCG	CCGACGACGT	TCCGGGGGGA	CCACCCGCCG	GCGAGGGTGA
8401	CGCGGGCAGC	GACGATGAGG	CTGCTGCCGG	CAGTCCTGGG	GTGTGGCCGT
8451	GGCTGGTGTC	GGCCAAGTCG	CAGCCGGCCC	TGCGCGCCCA	GGCCCAGGCC
8501	CTGCACGCCC	ACCTCACCGA	CCACCCCGGC	CTCGACCTCG	CGGATGTCGG
8551	ATACACCCTC	GCCCACGCCC	GCGCCGTGTT	CGACCACCGC	GCCACCCTCA
8601	TCGCCGCGGA	CCGCGACACG	TTCCTGCAAG	CACTCCAGGC	ACTCGCCGCA
8651	GGCGAGCCCC	ACCCCGCCGT	CATCCACAGC	AGCGCCCCGG	GCGGGACCGG
8701	GACCGGGGAG	GCCGCAGGAA	AGACCGCATT	CATCTGCTCC	GGACAGGGCA
8751	CCCAACGCCC	CGGCATGGCC	CACGGCCTCT	ACCACACCCA	CCCCGTCTTC
8801	GCCGCCGCAC	TCAACGACAT	CTGCACCCAC	CTCGACCCCC	ACCTCGACCA
8851	CCCCCTCCTC	CCCCTCCTCA	CCCAAAACGA	CAACGACAAC	GAGGACGCGG
8901	CCGCACTGCT	CCAGCAGACC	CGCTACGCCC	AGCCCGCCCT	CTTCGCCTTC
8951	CAGGTCGCCC	TCCACCGCCT	CCTCACCAGC	GGCTACCACA	TCACCCCCCA
9001	CTACTACGCC	GGACACTCCC	TCGGCGAAAT	CACCGCCGCC	CACCTCGCCG
9051	GCATCCTCAC	CCTCACCAGC	GCCACCACCC	TCATCACCCA	ACGCGCCACC
9101	CTCATGCAAA	CCATGCCCCC	CGGCACCATG	ACCACCCTCC	ACACCACCCC
9151	CCACCACATC	ACCCACCACC	TCACCGCCCA	CGAAAACGAC	CTCGCCATCG

Fig. 7e

9201	CCGCCATCAA	CACCCCCACC	TCCCTCGTCA	TCAGCGGCAC	CCCCCACACC
9251	GTCCAACACA	TCACCACCCT	CTGCCAACAA	CAAGGCATCA	AAACCAAAAC
9301	CCTCCCCACC	AACCACGCCT	TCCACTCCCC	CCACACCAAC	CCCATCCTCA
9351	ACCAACTCCA	CCAGCACACC	CAAACCCTCA	CCTACCACCC	ACCCACACC
9401	CCCCTCATCA	CCGACAACAC	CCCACCCGAC	CAACTCCTCA	CCCCCCTACTA
9451	CTGGACCCAA	CAAGCCCGCA	ACACCGTCGA	CTACGCCACC	ACCACCCAAA
9501	CCCTCCACCA	ACACGGCGTC	ACCACCTACA	TCGATCTCGG	ACCCGACAAC
9551	ACCCTCACCA	CCCTCACCCA	CCACAACCTC	CCCAACACCC	CCACCACCAC
9601	CCTCACCTC	ACCCACCCCC	ACCACCACCC	CCAAACCCAC	CTCCTCACCA
9651	ACCTCGCCAA	AACCACCACC	ACCTGGCACC	CCCACCACTA	CACCCACCAC
9701	CACAACCAAC	CCCACACCCA	CACCCACCTC	GACCTCCCCA	CCTACCCCTT
9751	CCAACACCAC	CACTACTGGC	TCGAAAGCAC	ACAGCCCGGT	GCCGGCAACG
9801	TGTCAGCAGC	CGGACTCGAC	CCCACCGAAC	ACCCCTACT	CGGCGCCACA
9851	TTGGAAGTGG	CGACTGACGG	TGGAGCGCTT	CTTGCAGGGC	GCTTGTCTTT
9901	GAGGTCGCAT	CCGTGGCTGG	CTGACCATGC	CGTCGGCGGC	ACGGTGCTGC
9951	TGTCGGGCGC	CACCTTCCTC	GAAGTCGCCC	TTCATGCGGG	CACATACGTG
10001	GGCTGCGACC	GAGTGGATGA	GCTGACGCTG	CATGCGCCGC	TGGTGGTTCC
10051	TGTGGATGGG	GGTGTGAGTG	TGCAGGTTGG	GGTTGCGGCT	GCGGATGGGG
10101	AGGGGCGGCG	TTTGGTGAGT	GTGTATGCGC	GGGGTGGGAG	TGCTTGTGGT
10151	GGGGGTGGTG	CGTCGGGTGG	GGTGTGGACG	TGTCATGCCT	CGGGGGTGCT
10201	GGTTGAGGCT	GCTGCTGGTG	GTGTGGTGCT	GGATGGTCTG	GCGGGGGTGT
10251	GGCCGCCGCG	GGGTGCGGTG	GCGGTGGATG	TCGATGGTGT	CCGTGACCGT
10301	TTGGCTGGGG	CTGGTTGTGT	TTTGGGGCCG	GTGTTTTTCGG	GGCTGCGTGC
10351	GGTGTGGCGT	GATGGGGGGG	ATTTGCTGGC	TGAGGTGTGT	CTGCCGGAGG
10401	AGGCGTGGGG	TGATGCGGCT	GGTTTTGGGC	TGCATCCGGC	GTTGCTGGAT
10451	GGTGTGGTCC	AGCCGTTGTC	GGTGTGCTT	CCGGGTGGGA	CGGGGTTTGG
10501	GGAGGGGGCG	GGGTTTCGGG	AGGGTGTTCG	GGTGCCGGCT	GTGTGGGGTG
10551	GTGTGTCGCT	TCACCGGGCG	GGTGTGACCG	GTGTGCGGGT	GCGTGTGTCTG
10601	GCTGTGCGGC	GGGGCGGCGG	GCGTGAGGCG	GTGTGCGGTCG	TGGTTCGGGA
10651	TGAGGCGGGT	GTGCCGGTGG	CGTCGGTCGA	TCGTCTTGAG	TTGCGGCCTG
10701	TGGATATGGG	TCAGTTGCGT	GCTGTCTCGG	TTTCGGCGGG	GCGGCGGGGT
10751	TCGCTGTATG	CGGTGCAGTG	GGCTGAGGTG	GGTCCTGTGC	CGGTGTGTGG
10801	GCAGGCGTGG	GCGTGGCACG	AGGACGTGGG	TGAGAGCGGT	GGTGGGCCTG
10851	TGCCGGGGGT	GGTGGTGTGG	CGGTGCCCGG	ATGCCGGTGC	CGGTGGCGGT
10901	GGCGGTGGCG	GTGCTGGCGG	TGGTGTGGGT	GAGGTTGTTG	GTGGGGTGTG
10951	GGGTGTGGTG	CAGGGGTGGC	TGGGGCTGGA	GCGGTTTGCG	GGTTCGCGGC
11001	TGGTGGTGGT	GACCCGGGGT	GCGGTGGTGG	CCGGCCCGGA	GGACGGCCCCG
11051	GTGGATGTGG	TGGGTGCGTC	GGTGTGGGGG	CTGGTGCGTT	CGGCGCAGGC
11101	TGAGCATCCG	GACCGGTTTG	TCCTCCTCGA	CCTCGACACC	GACACCGGCA
11151	CCGACCTCGA	CACCGGTGCT	GGTGTGTTG	GGGGCGTGGA	TGGTGGGCGT
11201	GTGGCGGCGG	TGGTGGCGTG	TGGTGAGCCG	CAGTTGGCGG	TGCGTGGGGA
11251	GCGGTTGCTG	GCCGCACGCC	TGACACGACT	TGAGTCATCC	GGTGATGTTT
11301	CAGCCCAGCG	GTCCGGTGAC	ACACGAGCCC	GGCGGTCCGA	CGTGCCTGCC
11351	CAGCGCTCCG	GTGGCGTGCC	TGCTCGGCGG	TCGGTTGATG	TATCGGGTGC
11401	GGAGGTGTTG	CCGTGGTTGT	CGGGTGGGTC	GGTGTGGTG	ACGGGTGGGA
11451	CGGGTGTGCT	GGGTGCGGCG	GTGGCGCGGC	ATCTGGCTGG	TGTGTGTGGG

Fig. 7f

11501	GTGCGGGATC	TGCTGTTGGT	GAGCCGGCGT	GGTCCGGATG	CTCCGGGTGC
11551	GGAGGGTCTG	CGGGCGGAGC	TGGCCGCGTT	GGGGGCGGAG	GTGCGGATTG
11601	TTGCGTGTGA	TGTGGGGGAG	CGGCGGGAGG	TGGTCCGGCT	GCTGGAGGGT
11651	GTTCCCTGCCG	GGTGTCCGCT	GACGGGTGTC	GTGCATGCGG	CTGGTGTGCT
11701	GGACGATGCG	ACGATCGCCT	CTCTCACGCC	CGAGCGGCTG	GGCACGGTGT
11751	TCGCGGCCAA	GGTGGATGCC	GCTCTTTTGC	TGGATGAGCT	GACGCGGGGT
11801	ATGGAGCTGT	CGGCGTTCGT	GCTGTTCTCC	TCGGCCGCGG	GGATCCTGGG
11851	GTCGGCCGGG	CAGGGCAACT	ACGCCGCGGC	CAATGCCGCT	CTGGACGCGC
11901	TGGCGTACCG	GCGGCGGGCG	GCGGGTCTGC	CGGGGGTGTC	GCTGGCGTGG
11951	GGGCTGTGGG	AAGAGGCCAG	CGGGATGACC	GGGCACCTGG	CCGGCACCGA
12001	CCACCGGCGC	ATCATCCGTT	CCGGTCTGCA	TCCCATGTGC	ACCCCGGACG
12051	CACTGGCCCT	CTTCGATGCG	GCCCTGGCTC	TGGACCGGCC	GGTCCTGCTG
12101	CCCGCCGACC	TGCGTCCCGC	CCCGCCCCTG	CCGCCCCTGC	TGCAGGACCT
12151	CCTGCCC GCC	ACCCGCCGCC	GCACCACCCG	CACCACCACT	ACCGGTGGTG
12201	CGGACAACGG	CGCCCAGCTG	CACGCCCGGC	TGGCCGGCCA	GACACACGAA
12251	CAACAGCACA	CCACCCTCCT	CGCCCTGGTC	CGCTCCCACA	TCGCCACCGT
12301	CCTGGGCCAC	ACCACCCCCG	ACACCATCCC	CCCCGACCGC	GCGTTCCGCG
12351	ACCTCGGCTT	CGACTCCCTC	ACCGCCGTGC	A	

DECLARATION, POWER OF ATTORNEY AND POWER TO INSPECT

As a below named inventor, I hereby declare:

that my residence, post office address and citizenship are as stated below next to my name;

that I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the invention entitled: **POLYKETIDES, THEIR PREPARATION, AND MATERIALS FOR USE THEREIN** the specification of which [check one(s) applicable]

X was filed 6 July 1999 as International Patent Application No. PCT/GB99/02158, on which U.S. National Stage Application No. 09/743,162 is based; and/or
— was amended by Amendment filed _____ (if applicable); and/or
— is attached to this Declaration, Power of Attorney and Power to Inspect;

that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and

that I acknowledge my duty to disclose information which is material to the examination of this application in accordance with Rule 56(a) [37 C.F.R. §1.56(a)].

CLAIM UNDER 35 U.S.C. §119: I hereby claim foreign priority benefits under 35 U.S.C. §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application of which priority is claimed:

<u>Prior Foreign Application(s)</u>	<u>Filing Date</u>	<u>Priority Claimed</u>
<u>Appln No.</u> <u>Country</u>	<u>Day-Mon-Year</u>	<u>Yes - No</u>
9814622.8 Great Britain	06-07-1998	Yes

POWER OF ATTORNEY: As inventor, I hereby appoint **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, Pennsylvania, and the following individual(s) as my attorneys or agents with full power of substitution to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith: **Patrick J. Hagan, Reg. No. 27,643** and **Kathleen D. Rigaut, Ph.D., Reg. 43,047**.

POWER TO INSPECT: I hereby give **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, Pennsylvania or its duly accredited representatives power to inspect and obtain copies of the papers on file relating to this application.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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